A STUDY OF *IN VITRO* OVULATION IN THE SPADEFOOT TOAD, *Scaphiopus holbrooki*

By

KEITH L. HANSEN

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TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF TABLES

LIST OF TABLES - Continued

LIST OF ILLUSTRATIONS

LIST OF PLATES

INTRODUCTION

The first experiments concerned with the induction of ovulation by the implantation of pituitary glands were performed on mammals (Aschein, 1926; Smith, 1926; Zondek, 1926). Since then many workers have demonstrated that pituitary materials will induce ovulation in representatives from most of the vertebrate classes (Osteichthyes: Houssay, 1931; Noble, 1936; Amphibia: Wolf, 1929; Rugh, 1934; Rostand, 1934; Shapiro, 1936; Reptilia: Houssay, 1930; Cunningham and Smart, 1934; Aves: Riddle and Flemion, 1928; Mammalia: Friedman, 1929; Hisaw et al., 1935, and many others).

Because of the simplicity of its ovarian structure and general availability, members of the Anura have been used widely in experimental studies of gonad-endocrine relationships. Structurally, the anuran ovary is essentially a hollow sac whose walls consist of a double membrane containing scattered smooth muscle cells and egg follicles. As the follicles mature, they stretch the inner cell layer and gradually project into the cavity within the ovary. In ovulation, the ovum passes through the thin outer membrane into the coelomic cavity. The process of ovulation and the anatomy of the ovary have been thoroughly described by Rugh (1935).

The early in vivo experiments involving pituitary injection and hypophysectomy revealed considerable information concerning

species specificity and seasonal periodicity in Amphibia (Houssay and Giusti, 1929; Adams, 1930; Adams and Granger, 1938; Creaser and Gorbman, 1939). Rugh (1937) presented quantitative data on differences in hormone potency in male and female gonadotropic factors, seasonal variation in the concentration of pituitary hormones, and variations in response by recipient females of different size and maturity.

In 1935 Rugh showed that excised ovaries from Rana pipiens would ovulate normally in Ringer's solution when their donors were injected with pituitary materials some hours before the removal of the ovary. In the first experiments exclusively employing the in vitro ovulation technique (Heilbrunn, Daugherty, and Wilbur, 1939), whole untreated ovaries were suspended in Ringer's solution containing macerated pituitary glands. Ovulation occurred in twelve to twenty hours, and all eggs so ovulated showed first polar body formation as do eggs ovulating naturally.

Ryan and Grant (1940) developed a slight modification of this technique by placing small pieces of ovary from Rana pipiens in Ringer's solution to which macerated pituitary concentrations had been added. These workers helped to establish the normalcy of in vitro ovulation by artificially fertilizing eggs which had been placed in the coelom of an ovariectomized female and allowed to pass through the oviducts to receive a jelly coat. Such eggs cleaved normally and many hatched into swimming larvae.

In 1945, Wright described some of the quantitative and biochemical aspects of *in vitro* ovulation in *Rana pipiens*. He showed that the ovulation response *in vitro* varied seasonally as Rugh (1937) had demonstrated *in vivo*. He also considered the effects of pituitary dosage, temperature, and the relationship of time to *in vitro* ovulation. Wright and Hisaw (1946) demonstrated that mammalian pituitary extracts (FSH and LH) elicit ovulation both *in vivo* and *in vitro*. A combination of FSH and LH produced ovulation in normal and hypophysectomized frogs as well as in ovarian fragments *in vitro*. Wright (1946) reported that hypophysectomy brought about an increased responsiveness of the ovary to pituitary substances *in vitro*. This temporary sensitization of the ovary was thought to be caused by the release of gonadotropins from the anterior lobe during the course of the operation. This hypothesis was strengthened by the increase of ovulation *in vitro* found following the immersion of ovarian pieces in serum taken from hypophysectomized frogs (Wright and Macintyre, 1950).

No *in vitro* ovulation studies have been done on the eastern spadefoot toad (*Scaphiopus holbrooki*). In fact, relatively little is known about this toad's reproductive physiology or breeding cycle. Field data indicate that this species is quite remarkable in its ability to breed over a wide number of months (January to October). It appears that the spadefoot does not follow a seasonal pattern of breeding like that of most Amphibia, but breeds at any time of the

year when environmental conditions are suitable. It has long been recognized by herpetologists and naturalists that this species is stimulated to breed by heavy rainfall during the warmer months. The exact mechanism by which this environmental stimulus affects the toad is unknown.

Using the technique of in vitro ovulation, it was hoped that this study might contribute toward a better knowledge of the physiological processes involved in ovulation as well as the neural-hormonal process involved in the breeding response of the spadefoot. The purpose of the present investigation was therefore twofold: first, to study a number of factors affecting ovulation in vitro, and second, to attempt to discover some of the physiological and ecological factors involved in the breeding of the spadefoot toad.

MATERIALS AND METHODS

Approximately 1007 spadefoot toads were used in the study. All were collected from a spacious dairy pasture located about six miles southwest of Gainesville, Florida. Collections were made at night, with the aid of a head light powered by a six-volt battery. Toads were easily located by the reflection of the light from their eyes.

Following collection, the sexes were separated in the laboratory. Spadefoots were placed in square-shaped gallon jars, each containing ten to twelve toads. A thin film of water in the bottom protected the animals from desiccation. The water was changed periodically. The jar lids were set on loosely to allow sufficient fresh air. The spadefoots were stored in a refrigerator set at 12° C. where they could be kept in a state of dormancy for several months without feeding. Most toads, however, were used during the same month in which they were collected.

In general, the laboratory procedures were similar to those described by Wright (1945). The ovarian donors were moderate to large females weighing from 10 to 22 grams and having head widths varying from 18 to 23 mm. It was found that no relationship existed between the size of the female and the size or maturity of the eggs. Thus a wide range of sizes could be utilized. By pressing upon the abdominal wall and pushing one ovary to the transparent groin area,

each female was examined for a full complement of mature eggs. In this way, immature or barren females were culled rather than needlessly sacrificed.

The ovaries (Pl. I, fig. 1), which are similar to those of other anurans in structure, were removed from freshly killed females and placed in a Petri dish containing 30 ml. of Holtfreter's solution (normal solution), which is approximately isotonic with amphibian embryonic tissues (Holtfreter, 1945). The ovaries were later removed from the solution, weighed, and then cut into small pieces containing twenty to thirty-five eggs each. These fragments were suspended by cotton threads in stoppered vials containing 10 ml. of Holtfreter's solution (Pl. I, figs. 2 and 3). Pituitary homogenate, usually prepared from the anterior lobes (pars distalis) of adult male toads, was added to this fluid to induce ovulation.

The extirpation of the pituitary gland was relatively simple. By means of a quick cut with scissors through the angle of the mouth and across the cervical region, the entire cranium was removed from the body and lower jaw. A second transverse cut removed the remaining cervical vertebrae and exposed the foramen magnum (Pl. II, fig. 1). The skin covering the roof of the mouth was pulled off anteriorly with a pair of heavy forceps (Pl. II, fig. 2). Each lateral projection of the exposed T-shaped parasphenoid bone was cut with a pair

of fine-pointed scissors. Then one blade of the scissors was inserted into the foramen magnum, lateral to the medulla, and, with the other blade resting upon the lateral process of the parasphenoid bone, a single cut was made along either side of the ventral surface of the brain case. The bony flap thus formed was folded anteriorly, exposing the ventral surface of the brain with the pituitary gland attached to the infundibulo-tuberal region of the hypothalamus, just posterior to the optic chiasma (Pl. II, fig. 3). The pars distalis is a round glandular portion of the hypophysis and is easily removed from the pars nervosa (posterior lobe) and pars intermedia (intermediate lobe) with fine-pointed surgical forceps.

Since a standardized potency of pituitary suspension was needed, given numbers of male anterior lobes were finely macerated with the use of a small mortar and pestle. The macerated homogenate was then removed and diluted in an appropriate amount of Holtfreter's solution. The ratio of pituitary to normal fluid was one anterior lobe to 10 ml. of Holtfreter's solution.

Blank controls of normal fluid, without pituitary homogenate, were used in every experiment to check for possible spontaneous ovulation. All experiments were allowed to stand for eighteen hours, and unless otherwise indicated, all tests were conducted at a room temperature of 23.0 $\pm$ 1.0° C.

Percentages of ovulation were determined by dividing the number of eggs ovulated (i.e., falling to the bottom of the vial) by the total number of eggs present in the ovarian fragment, and multiplying by 100. All statistical comparisons were based upon the ovulation percentages. A complete sample of data may be seen in Table 10.

After data had been obtained on in vitro ovulation, female toads were subjected to certain stimuli (e.g., lowered atmospheric pressure, presence of excess water, etc.) before being sacrificed for their ovaries in an attempt to determine whether such stimuli might play a role in the breeding of the spadefoot toad in nature. Since the experimental procedures varied, a detailed description of these is given along with the results.

PROCEDURES AND RESULTS

Factors Affecting In Vitro Ovulation

The following aspects of *in vitro* ovulation in the spadefoot toad were considered in this work: individual variation in ovulation; size relationships in pituitary and ovarian donors; comparative effects of male and female pituitary; influence of varying concentrations of pituitary suspensions; and, influence of heteroplastic pituitary implants. The effects of certain physical factors such as light, temperature, and pH, and the relationship of time to ovulation *in vitro* were also studied.

Comparative Potency of Male and Female Pituitary

Both Wright (1945) and Rondell (1953), working on *in vitro* ovulation in *Rana pipiens*, used pituitary homogenates prepared from the anterior lobes of female frogs only. Since all toads used in this study were collected by the writer, it seemed desirable to utilize the males (approximately fifty percent of the individuals taken) as a source of pituitary. However, the question arose concerning the relative potency of the male and female glands since, in *Rana pipiens*, Rugh (1937) found the pituitary of the male to be 16 percent heavier than but only 60 percent as potent as that of the female.

Two tests were designed to compare the relative potency of the male and female glands. In the first test pituitaries were removed from six males and six females of equal size. The glands were placed on two tared cover-slips and dried in an oven held at 60° C. for twenty-four hours. Following the second weighing, appropriate dilutions were made to give a twelve-vial series for each sex. In each vial the equivalent of one-half of an anterior lobe was suspended in 5.0 ml. of Holtfreter's solution. The effectiveness of these dilutions in inducing in vitro ovulation was then tested with the results shown in Table 1, and the male pituitary was found to be 75.7 percent as potent as that of the female.

The second test utilized freshly triturated pituitary glands from five males having a mean head width of 20.9 mm., and five females with a mean head width of 20.8 mm. Pituitary homogenates of the glands of each sex were prepared and distributed equally in ten vials, each containing 5.0 ml. of fluid. The ovulation results for the freshly macerated glands are recorded in Table 1. In the second experiment, the male pituitary proved to be 81.0 percent as potent as the female gland. If a mean percentage is taken for the two experiments, the male glands are 78.2 percent as potent as the female glands. This is appreciably greater than the 60 percent reported for Rana pipiens by Rugh (1937). A histogram presents the results for Scaphiopus holbrooki in Figure 1.

TABLE 1

COMPARATIVE POTENCY OF MALE AND FEMALE PITUITARY

Type of Pituitary Preparation	Sex	Percent Ovulation in Ovarian Fragments*							Mean	Relative Potency**
		Control (normal fluid)	Experimental (1/2 pit. in 5.0 ml. of normal fluid)							
Desiccated Glands	Female	0.0	31.9 34.6	28.4 22.7	22.2 20.1	18.2 18.2	12.0 13.2	8.5 12.5	20.2	100.0
	Male	0.0	31.4 20.4	16.7 18.7	16.7 16.9	12.2 13.5	7.4 11.9	7.1 10.2	15.3	75.7
Fresh Triturated Glands	Female	0.0	76.5 47.8	74.2 47.4	59.1 42.3	54.2 40.0	52.0 34.6		52.8	100.0
	Male	0.0	76.2 54.5	66.7 21.1	61.5 18.8	56.5 10.0	56.3 6.3		42.8	81.0

*For explanation of method used in determining percentage ovulation, see page 8 or Table 10.

**The potency of the female pituitary was arbitrarily assigned a value of 100% for comparative purposes.

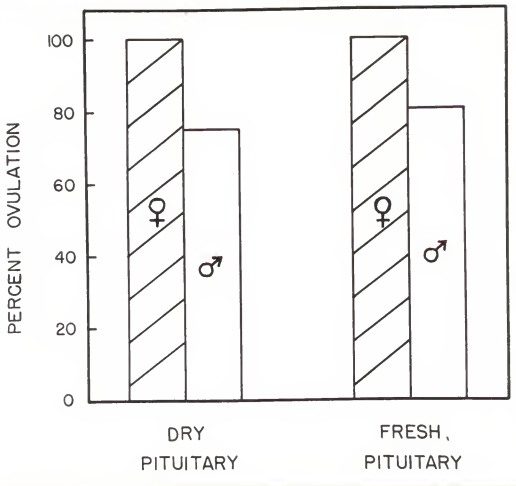

Fig. 1. A comparison of the potency of male and female pituitary glands determined by the in vitro ovulation technique. For comparative purposes, an arbitrary value of 100% was assigned to the potency of the female glands.

Since males were readily available from field collections, and since the potency of male anterior pituitary lobes was sufficient to produce a high percentage of ovulation, male pituitary was used almost exclusively throughout the present work.

Individual Variation in Ovulation In Vitro

Both Wright (1945) and Rondell (1953), using Rana pipiens, found considerable variation in ovulation using the in vitro technique. To give statistically satisfactory results, Rondell used a minimum of four pieces from random areas of the ovary of each of two frogs. In such an eight-vial series receiving the same treatment, the variation in percentage of ovulation never exceeded 15 percent.

To test the variation in percentage of ovulation among individuals, in the present study, six females of approximately the same size were used. Eight pieces of ovary were taken from each toad and suspended in vials containing the equivalent of one-half male pituitary in 10 ml. of solution. The individual and mean percentages of ovulation are recorded in Table 2 along with the standard deviation and standard error of the means. When Rondell's method of using four pieces of ovary from each of two frogs was applied, the greatest variation between extreme means proved to be 15.2 percent which is almost identical to that found by Rondell.

TABLE 2

INDIVIDUAL VARIATION OF IN VITRO OVULATION

Toad Number	Percent Ovulation With One-half Pit. in 10 Ml. Fluid								Mean	Stand. Dev.	Stand. Error
1.	79.5	66.7	43.2	40.0	86.2	43.3	70.9	54.1	60.6	17.3	6.1
2.	37.5	78.3	85.2	73.7	21.7	66.7	75.0	53.8	61.2	20.6	7.3
3.	53.8	36.7	47.4	48.4	75.0	63.3	60.5	44.4	53.1	10.9	3.8
4.	79.3	59.3	45.0	30.6	44.1	62.5	60.0	45.4	53.1	12.9	4.6
5.	48.4	48.3	68.2	30.7	40.0	70.0	46.7	63.3	51.9	13.3	4.7
6.	64.3	40.0	37.5	67.6	41.0	60.0	61.8	62.1	54.1	11.6	4.1

However, in order to have a still further check on the validity of this technique, the "t." test for statistically significant differences was applied to the means of the percentages of ovulation. The results shown in Table 3 indicate that the differences between the means lack statistical significance. It may be concluded, therefore, that individual variation in ovulation for toads subjected to the same conditions, is not great enough to produce statistically significant differences. Therefore, statistical comparisons, showing significant differences for toads exposed to varying conditions, may be relied upon as valid.

Relationships of Toad Size to *In Vitro* Ovulation

Size of Pituitary Donors. It seemed logical to assume that larger animals possessed proportionately larger pituitary glands and that these probably had a greater hormone titer. To test this assumption, lobes from twenty-one male toads ranging in head width from 17 to 23 mm. were selected. Their anterior lobes were removed, macerated individually, and suspended in 10 ml. of Holtfreter's solution. Each of these 10 ml. quantities was divided equally between two vials. In this manner, the potency of the pituitary of each toad was checked with two pieces of ovary. The ovary, taken from a single female, was divided among the forty-two vials of pituitary solution. The mean percentage of ovulation for each size

TABLE 3

"T" TESTS FOR STATISTICALLY SIGNIFICANT
DIFFERENCES BETWEEN MEAN OVULATION PERCENTAGES

Toads	1.	2.	3.	4.	5.	6.
1.	-	-	-	-	-	-
2.	0.06	-	-	-	-	-
3.	0.90	0.92	-	-	-	-
4.	0.91	0.88	0.00	-	-	-
5.	1.05	1.00	0.15	0.18	-	-
6.	0.83	0.79	0.16	0.15	0.33	-

class was as follows: 23mm.) - 35.7%; 22 mm.) - 37.2%; 21 mm.) - 32.5%; 20 mm.) - 28.7%; 19 mm.) - 36.1%; 18 mm.) - 36.7%; and 17 mm.)- 28.9%. The individual ovulation results are shown in Table 4.

The means of the various size classes showed only minor differences, and the coefficient of correlation ($r = + 0.11$) between the size of pituitary donors and percentage of ovulation was quite low; this lacked statistical significance (Table 7) because of the small numbers involved. From this experiment, it appears that the size of pituitary donors (actually the size of their pituitaries) has a negligible effect upon in vitro ovulation.

Since careful body measurements were taken of the male and female toads used in all experiments, the relation of the pituitary size to ovulation could be studied further. It was possible to take the ovulation data from all of the experiments (350 individual tests) in which female toads were not subjected to experimental conditions (Table 5). Again, a correlation value was sought. The coefficient of correlation calculated from these data gave a low negative value, where $r = - 0.16$. This test proved to be statistically significant with $t. = 3.06$, $P. < 0.05$ (Table 7).

The data given in Table 5 present a rather complex picture. The means of the percentages of ovulation for each size class, at the different pituitary dilutions, were compared statistically.

TABLE 4

RELATIONSHIP OF PITUITARY DONOR SIZE TO IN VITRO OVULATION

Toad Number	Percentages of Ovulation for Size Classes of Male Pituitary Donors						
	Head width in millimeters						
	23	22	21	20	19	18	17
1.	33.9	51.2	39.2	27.3	31.8	55.8	31.9
2.	38.0	16.9	32.1	23.1	39.3	17.6	26.5
3.	35.1	43.6	25.8	35.6	37.2	36.6	28.4
Mean	35.7	37.2	32.5	28.7	36.1	36.7	28.9

TABLE 5

RELATIONSHIP OF MALE PITUITARY DONOR SIZE TO IN VITRO OVULATION

Toad Head Width Size	Percent Ovulation Obtained From Pituitary Dilutions													
	Number of anterior lobes in 10 ml. fluid													
	1		1/2		1/4		1/8		1/16		N	Mean	Stand. Dev.	Stand. Error
	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean				
22	3	37.2	3	17.6	3	16.7	3	13.7	2	8.9	14	19.6	14.9	4.0
21	8	27.3	6	37.4	6	29.7	6	16.4	4	9.5	30	24.5	18.7	3.4
20	19	30.3	28	26.6	27	18.9	26	9.8	8	4.1	108	19.6	17.6	1.7
19	24	45.4	31	24.1	25	17.7	27	8.4	7	7.7	114	21.8	21.5	2.0
18	39	28.6	7	43.2	7	25.6	3	21.7	3	3.9	59	27.2	19.2	2.5
17	6	37.5	5	20.5	5	22.4	5	9.3	4	6.7	25	19.5	15.3	2.8

Table 6 shows the various comparisons, using the "t." test, between the size group means. Only that between the 18 and 20 mm. groups shows statistically significant differences. It would appear, therefore, that the size of the pituitary donor is not correlated with the percentage of ovulation induced.

Size of Ovary Donors. In the preliminary stages of the work, it was learned that small, yolk-deficient eggs seldom ovulated normally, therefore, immature eggs were avoided. It seemed desirable, however, to determine whether there was any correlation between the size of toads and the maturity of their eggs.

Females varying in head width from 17 to 23 mm. were divided into seven size classes. Two females were selected for each size class, and two pieces of ovary were used for each of these. Each size class was therefore represented by a four-vial series in which each vial contained the equivalent of one-half pituitary in 5 ml. of fluid. The individual and mean values for percentage of ovulation in the various classes are shown in Table 8. The correlation between size of female and percentage of ovulation appears to be practically nil, and this is supported by a statistical analysis. The coefficient of correlation proved to be very low ($r = + 0.06$) although not significant statistically (Table 7) because of the small numbers involved.

TABLE 6

VALUES (t.) FROM TESTS FOR STATISTICALLY SIGNIFICANT DIFFERENCES BETWEEN OVULATION PERCENTAGES INDUCED BY PITUITARIES FROM DIFFERENT SIZED DONORS

Male Toad Head Width Size	17	18	19	20	21	22
17	-	-	-	-	-	-
18	1.98	-	-	-	-	-
19	0.68	1.65	-	-	-	-
20	0.05	2.48	0.42	-	-	-
21	1.13	0.62	0.67	1.26	-	-
22	0.04	1.55	0.47	0.005	0.90	-

TABLE 7

COEFFICIENT OF CORRELATION VALUES BETWEEN DONOR TOADS AND PERCENT OVULATION

Tests	r	Standard Deviation	t	P
Test between size of pituitary donors (20 males) and ovulation induced	+ 0.11	0.22	0.48	Not Sign.
Test between size of pituitary donors (350 males) and ovulation	- 0.16	0.05	3.06	P.<0.05
Test between size of ovary donors and percent ovulation	+ 0.06	0.19	0.32	Not Sign.

TABLE 8

RELATIONSHIP OF OVARY DONOR SIZE TO IN VITRO OVULATION

Toad Number	Percentages of Ovulation for Size Classes of Ovary Donors						
	Head width in millimeters						
	23	22	21	20	19	18	17
1.	86.4	70.6	74.7	59.2	71.2	80.4	57.2
	75.2	65.0	65.1	67.4	48.4	73.0	52.0
2.	61.1	33.0	57.9	38.1	22.1	60.1	62.0
	32.3	27.2	67.2	41.9	24.8	42.1	43.2
Mean	63.5	46.5	59.9	51.7	41.6	63.9	53.6

Effects of Pituitary Dilutions Upon *In Vitro* Ovulation

Wright (1945) found that one-eighth of an anterior lobe suspended in 10 ml. of Holtfreter's solution produced maximum *in vitro* ovulation in *Rana pipiens*, but it was necessary to establish the optimal amount of pituitary necessary to produce approximately maximal ovulation in the spadefoot. For this purpose, forty-nine male pituitary donors and three female ovary donors were selected. To avoid individual variation in potency, a standardized stock solution of pituitary was prepared. The desired concentrations (from six glands to one sixty-fourth of a gland per 10 ml. of Holtfreter's solution) were obtained by mixing together appropriate amounts of this stock solution and of Holtfreter's solution. The records of ovulation and the mean values for each pituitary dilution are found in Table 9. The curve in Figure 2 shows these data in graphic form. The ovulation percentages for the various pituitary dilutions are as follows: six pituitaries in 10 ml. of fluid - 7.5 percent; five - 28.5 percent; four - 44.4 percent; three - 59.5 percent; two - 61.8 percent; one - 64.5 percent; one-half - 42.1 percent; one-fourth - 22.5 percent; one-eighth - 11.1 percent; one-sixteenth - 4.3 percent; one thirty-second - 1.4 percent; one sixty-fourth - 0.0 percent.

TABLE 9

EFFECTS OF PITUITARY DILUTIONS UPON IN VITRO OVULATION

Female Ovary Donor	Percent Ovulation with the Following Number of Male Pituitaries in 10 ml. of Fluid											
	6	5	4	3	2	1	1/2	1/4	1/8	1/16	1/32	1/64
1.	7.5	19.2	28.6	39.2	50.5	55.8	39.0	19.3	8.8	3.1	1.1	0.0
2.	-	37.7	50.0	63.1	58.1	52.7	42.4	17.5	7.7	2.0	0.0	0.0
3.	-	-	54.7	76.2	76.7	85.7	44.9	30.7	16.8	7.8	3.1	0.0
Mean	7.5	28.5	44.4	59.5	61.8	64.7	42.1	22.5	11.1	4.3	1.4	0.0

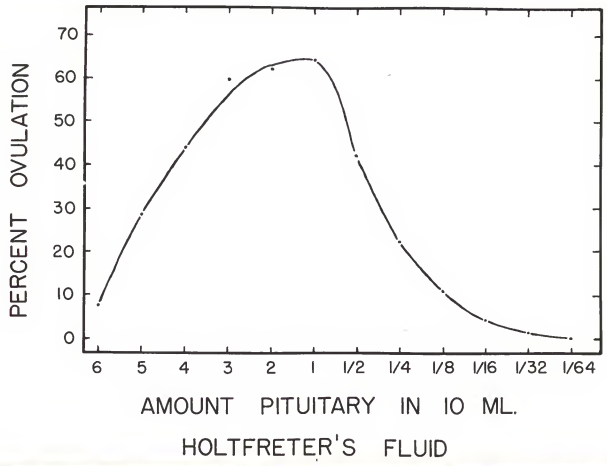

Fig. 2. In vitro ovulation results obtained from various concentrations of pituitary suspensions. Each point on the graph represents the average percentage of ovulation obtained in three vials.

From this experiment it was found that one pituitary in 10 ml. of fluid produces essentially maximal ovulation. It is also evident from these results that ovulation is inhibited at higher pituitary dilutions, which is in accordance with Wright (1945) who found inhibition of ovulation in Rana pipiens when using greater concentrations of pituitary. The results of the present study are similar to those of Foster, Foster, and Hisaw (1937) who found that ovulation in mammals may not follow the administration of larger doses of an unfractionated pituitary preparation.

Relationship of Time to In Vitro Ovulation

In order to ensure the accuracy of the results, it was necessary to determine the time required for the ovulation process to reach a stage where eggs were no longer ovulated. Wright (1945), using Rana pipiens, performed a time experiment at room temperature (22° C.) with dilutions of one-eighth of a pituitary in 10 ml. of Holtfreter's solution. He found that ovulation did not start until about the tenth hour, and that when dilute pituitary dilutions were used (i.e., one sixty-fourth or one, one hundred twenty-eighth pituitary in 10 ml. of fluid), ovulation did not begin for sixteen to eighteen hours.

Four different tests were conducted in the current work. Each employed a four-vial series. Each vial contained a pituitary

homogenate equivalent to one pituitary in 10 ml. of solution. The hourly progress of ovulation is shown in Figure 3. From these four curves it may be seen that in vitro ovulation began about three and one-half hours after the ovarian fragments were placed in the solution. Once the process was initiated, ovulation was most rapid in the following three hours when approximately seventy-five percent of the eggs ovulated. Ovulation was completed in all experiments after eight and one-half to eleven and one-half hours.

This series of experiments also showed that maximal ovulation occurred during the sixth hour. A curve, the points of which represent the ovulation percentages for each hour, presents these data in Figure 4. Wright (1945) found, however, that this took place between the thirteenth and seventeenth hours in Rana pipiens using a one-eighth pituitary dilution.

Wright's study further indicated that lower dilutions of pituitary took longer to initiate ovulation. In order to test this for Scaphiopus holbrooki, a series of five pituitary dilutions were prepared, using from one to five glands in 10 ml. of fluid. For each concentration, 5 ml. of solution were placed in each of two vials so that a double test might be used. The results are presented graphically in the form of five curves representing the different pituitary concentrations (Fig. 5). The four-pituitary solution

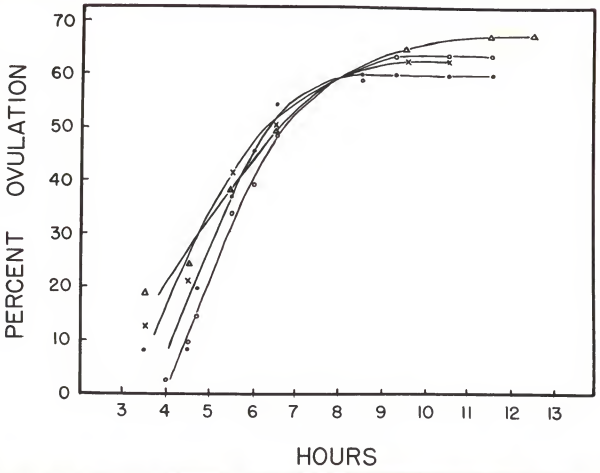

Fig. 3. Progress of <u>in vitro</u> ovulation from four different time tests. Each point on a curve represents the average ovulation percentage from a four-vial series.

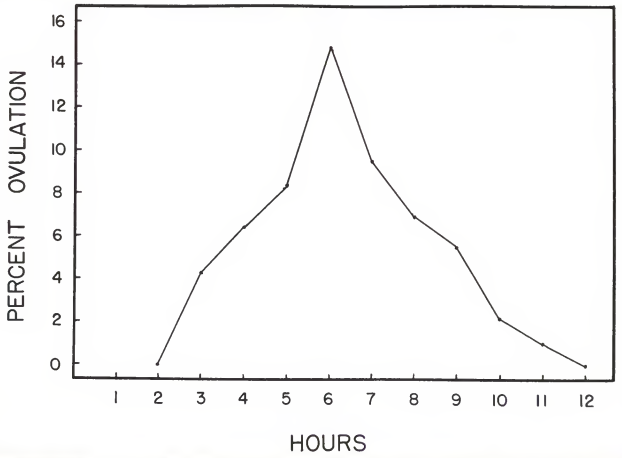

Fig. 4. Percentage of <u>in vitro</u> ovulation recorded hourly. The peak in ovulation occurred in the sixth hour.

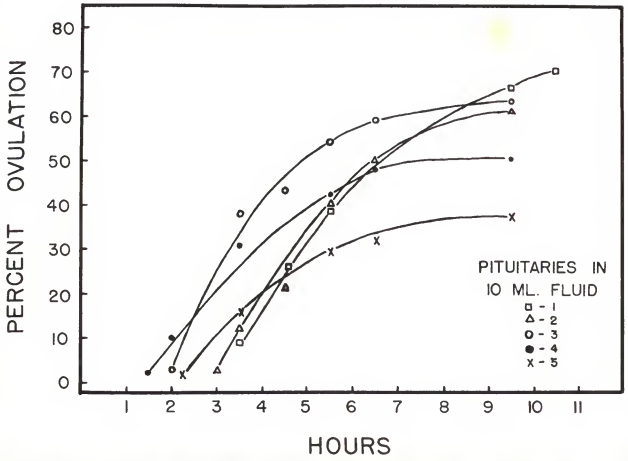

Fig. 5. Effect of pituitary dilutions upon the time required to initiate in vitro ovulation. It should be noted that the lower dilutions were more effective in inducing ovulation.

initiated the ovulatory process in one and one-half hours, the three-gland solution in two hours, the five-gland in two and one-fourth hours, the two-gland in three hours, and the one-gland in three and one-half hours. Thus it appears that, up to a point, higher pituitary concentrations initiate the ovulatory response in less time. However, at the five-gland level, the initiation of ovulation was somewhat retarded. This action is probably due to the inhibitory stimulus found in higher concentrations of pituitary (see section on Pituitary Dilutions). Pituitary concentrations of one, three, two, four, and five anterior lobes produced in order, highest to lowest final percentages of ovulation.

Effect of Light Upon In Vitro Ovulation

Since experiments were to be carried on at all hours of the day, it seemed necessary to determine whether the presence of light affected in vitro ovulation. A standardized pituitary suspension was prepared for an eight-vial series, each vial containing the equivalent of one-half of an anterior lobe in 10 ml. of normal solution. The ovary from a single female was cut into small pieces under a Wratten Safelight (Series OA) in a photographic darkroom. Four control vials were left in this room in absolute darkness. Four experimental vials were removed to an adjacent room of the same temperature (78° C.) and there exposed to the light of a 100 watt

bulb placed 12 inches from the vials. White paper was placed back of the vials to throw reflected light to all parts of the suspended pieces of ovary. Each set of vials was placed in a large shallow pan of water so that no significant temperature fluctuations would take place.

The results from this experiment are shown in Table 10. A statistical analysis was made of these data and the difference in ovulation percentages was found to lack statistical significance ($t. = 1.01$, $P. > 0.05$). It was therefore concluded that light plays no significant role in the stimulation or inhibition of the ovulation process *in vitro*.

Effect of Temperature Upon *In Vitro* Ovulation

Wright (1945) conducted a test to determine the effect of temperature on *in vitro* ovulation in *Rana pipiens*. He found that *in vitro* ovulation took place between temperatures of 17° and 32° C., with an optimum at about 22° C. and that no ovulation occurred at 12° or 37° C. He did not, however, establish definite temperature limits for the process.

Four experiments were undertaken to test the effects of temperature on *in vitro* ovulation in *Scaphiopus holbrooki*. Each of the experimental vials contained the equivalent of one-half of

TABLE 10

EFFECT OF LIGHT ON IN VITRO OVULATION

Treatment		Vial Series (1/2 pituitary in 10 ml. fluid) 1	2	3	4	Total Number of Eggs	Total Eggs Ovulated	Mean Percentage Ovulation	Test of Significance
Exposed to Light	Eggs ovulated / Eggs remaining in ovarian fragment	4/34	6/22	5/25	3/17	116	18	16.0±1.81	
	Percentage Ovulation	10.5	21.4	16.7	15.0				t. = 1.01 P. > 0.05
Kept in Darkness	Eggs ovulated / Eggs remaining in ovarian fragment	5/22	3/40	5/27	3/28	125	16	12.5±2.39	
	Percentage Ovulation	18.5	7.0	15.6	9.7				

a pituitary in 10 ml. of Holtfreter's solution, and a single ovary donor was used. Three refrigerators and an incubator were used to achieve the desired range of temperatures. Before immersing the ovarian fragments in the pituitary solutions, the vials were allowed to attain the temperature at which they were to be maintained throughout the course of the experiment. The first experiment was designed to give a wide range of temperatures (4 - 35° C.), in order that approximate temperature limits might be ascertained. Subsequent experiments were refined to determine the exact upper and lower limits, as well as the optimum temperature.

The individual and mean results for each temperature class are presented in Table 11. From these data it may be concluded that *in vitro* ovulation occurs between 10° and 30° C. with an optimum at about 24° C. The percentage of ovulation is reduced at both lower (10 - 20° C.) and higher temperatures (28 - 30° C.), and complete inhibition occurs at 9° C. and at 31° C. A temperature curve based on these data is shown in Figure 6.

Effect of pH on *In Vitro* Ovulation

Rondell (1953), using *Rana pipiens*, found that *in vitro* ovulation took place between pH values of 6.6 and 8.2, with complete inhibition at 6.0. Very little is known concerning the effects of

TABLE 11

EFFECT OF TEMPERATURE ON IN VITRO OVULATION

Experiment Number	Percent Ovulation at the Various Temperatures, Using One-half Pituitary in 10 ml. Fluid										
	4° C.	9° C.	10° C.	12° C.	15° C.	20° C.	24° C.	28° C.	30° C.	31° C.	35° C.
1.	0.0	-	-	10.2	-	20.5	26.5	-	3.6	-	0.0
2.	-	-	-	40.6	41.3	48.5	53.1	37.8	-	-	-
3.	-	0.0	12.8	-	9.8	-	36.1	-	-	0.0	-
4.	-	0.0	-	17.0	-	-	41.9	39.5	8.9	0.0	-
Mean	0.0	0.0	12.8	22.6	25.6	34.5	39.4	38.7	6.3	0.0	0.0

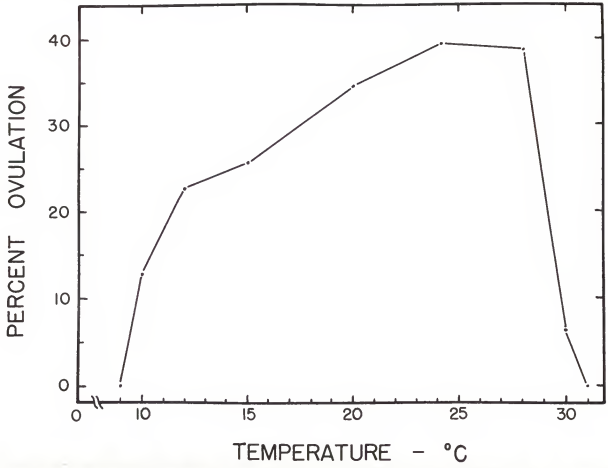

Fig. 6. In vitro ovulation after exposure of ovarian fragments to a range of temperatures.

pH upon the biocatalytic action of hormones. Since the buffering capacity of Holtfreter's solution is slight, it seemed important to determine the influence of pH on the ovulation process in vitro.

Two biological buffering systems were employed. The first was a veronal acetate buffer (Gortner and Gortner, 1949), prepared by adding 14.71 grams of sodium veronal and 9.71 grams of sodium acetate trihydrate to 500 ml. of distilled water. To a given amount of this solution, various quantities of 0.1M HCL were added, producing a pH range from 3.01 to 9.42. Using this buffering system, in vitro tests were run at pH values ranging from 4.01 to 9.42. Two vials were used for each pH value. The pituitary homogenate was added to the stock solution of veronal acetate. Both experimental and control vials contained the equivalent of one-half of a pituitary in 10 ml. of buffer or Holtfreter's solution, respectively. A second control was used in which the vials contained only Holtfreter's fluid with no pituitary.

All experimental vials (those with buffer and pituitary) gave negative results, as did the control blanks containing Holtfreter's solution without pituitary. The control vials with pituitary ovulated normally, however, with a mean ovulation percentage of 38.7 percent. Thus ovulation was inhibited by this particular buffer system, probably because of the narcotic effects of the sodium veronal, which is a barbiturate derivative.

Since veronal acetate obviously could not be used as a buffer in these experiments, a phosphate buffering system was prepared in which the pH was adjusted by varying the ratio of monobasic sodium phosphate ($NaH_2PO_4.H_2O$) to dibasic sodium phosphate ($Na_2HPO_4.7H_2O$), after Gomori (1952). In preliminary tests ovulation was found to proceed normally at 0.1M and 0.07M concentrations of the phosphate buffer, but, at molarities of 0.007M and lower, all eggs were cytolyzed due to the hypotonicity of the buffer solution. Since Holtfreter's solution has a molarity of 0.0638M, a molarity of 0.07M was maintained for the phosphate buffering system, but the ratio of monobasic to dibasic sodium phosphate was varied to produce for the tests a pH range of 5.9 to 8.9. For a further check, each solution was tested with the Beckman pH meter after the addition of the pituitary homogenate. The pituitary homogenate was added to a small quantity of the dibasic solution and this was distributed equally among all vials. Additional dibasic solution was added to those vials requiring higher ratios of the dibasic to monobasic phosphate.

The results from these five tests are recorded in Table 12, and are presented in the form of a curve in Figure 7. From these findings it is evident that ovulation occurs over a relatively wide range of hydrogen ion concentrations with a peak of activity at a pH of 7.3. It is inhibited, however, below 6.5 and above 8.5.

TABLE 12

EFFECT OF PH ON IN VITRO OVULATION

Tests	Percent Ovulation Occurring in Phosphate Buffer At Various PH Values - One-half Pituitary in 10 Ml. of Fluid																Percent Ovulation for Controls	
	pH Values																With Pit.	Without Pit.
	5.9	6.1	6.3	6.5	6.7	6.9	7.1	7.3	7.5	7.7	7.9	8.1	8.3	8.5	8.7	8.9		
1.	-	-	0.0	4.7	9.3	2.9	-	-	-	-	3.0	8.2	6.3	3.7	0.0	-	15.6	0.0
2.	-	-	-	0.0	0.0	-	-	-	-	-	2.2	2.3	4.1	2.3	0.0	0.0	11.1	0.0
3.	-	-	-	0.0	9.1	-	19.8	27.1	24.2	-	45.8	-	-	-	-	-	34.0	0.0
4.	0.0	0.0	0.0	-	3.9	28.3	22.6	29.8	-	20.4	31.6	-	-	-	-	-	41.4	0.0
5.	0.0	0.0	0.0	-	-	-	-	-	10.0	11.6	10.0	-	-	-	-	-	20.5	0.0
Mean	0.0	0.0	0.0	1.6	5.6	15.6	21.2	28.5	17.1	16.0	18.5	5.3	5.2	3.0	0.0	0.0	24.5	0.0

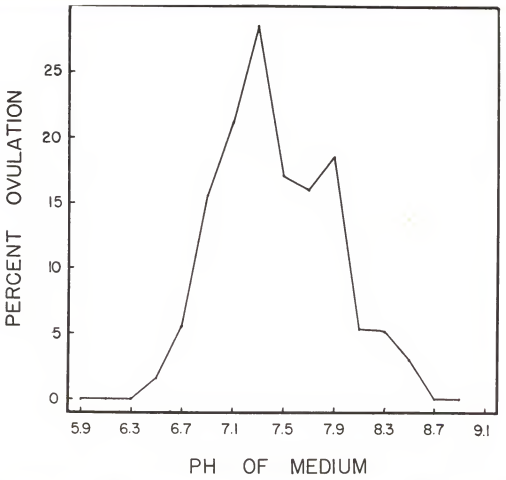

Fig. 7. Effect of hydrogen ion concentrations upon in vitro ovulation. Maximal ovulation was obtained near the point of neutrality (7.3).

Effects of Heteroplastic Pituitary Materials Upon *In Vitro* Ovulation

Wolf (1929) conducted the first experiments on the induction of ovulation in Amphibia by the implantation of pituitary glands. Since that date numerous similar experiments have been performed using both homoplastic and heteroplastic tissues. Creaser and Gorbman (1939), after reviewing the literature on induced ovulation among Amphibia, brought forth the following important generalizations: 1) that Amphibia and other classes of vertebrates respond readily to very slight amounts of homoplastic pituitary materials; 2) that the effectiveness of a gonadotropic hormone from a foreign species tends to vary directly with the phylogenetic affinity of the donor and recipient species; 3) that a qualitative specificity exists in gonadotropic hormones; and, 4) that the variation in gonad specificity between widely separated donor and recipient species may be great enough to lead to an apparent ineffectiveness of the hormone.

Because of the correlation between gonadotropin specificity and the phylogenetic relationship, a study was made of the effects of heteroplastic pituitary materials upon the *in vitro* process. Since the size and hormone titer of the various pituitaries were quite variable, it was impossible to utilize the results for quantitative comparisons, and they are therefore listed simply as positive or negative. It is likely that this ovulatory technique might be used for precise quantitative measurements of hormonal homologies.

An attempt was made to secure representative pituitary donors from the majority of vertebrate classes. Particular attention was given to the relationships within the Amphibia. The comparative results are recorded in Table 13, while a diagrammatic phylogenetic tree shows where the different effects took place (Fig. 8). Control vials, each containing one male spadefoot pituitary suspended in 10 ml. of fluid, were used in all instances. From this study it is seen that the in vitro ovulation mechanism is quite sensitive to heteroplastic pituitary implants. Pituitary materials from amphibians, reptiles, birds, and most mammals evoked ovulation in vitro. Only the piscine pituitary, swine gonadotropic fraction, and human pregnancy urine failed to induce ovulation. Thus, it is evident that pituitary materials from Amphibia and higher phylogenetic categories stimulate the eggs of Scaphiopus to ovulate in vitro.

TABLE 13

EFFECTS OF HETEROPLASTIC PITUITARY MATERIALS UPON IN VITRO OVULATION

Source of Pituitary Preparation	Nature of Preparation	No. Pit. Glands in 10 ml. Fluid	Percentage of Ovulation		General Results
			Heteroplastic Pituitaries	Controls (1 Spadefoot in 10 ml. Fluid)	
Osteichthys					
Cynoscion nebulosus (Weak Fish)	Whole Pit. Fresh Homogenate	5	0.0	45.6	Negative
		3	0.0		
		2	0.0		
		1	0.0		
Sciaenops ocellatus (Channel Bass)	Whole Pit. Fresh Homogenate	5	0.0	45.6	Negative
		3	0.0		
		2	0.0		
		1	0.0		
Amphibia					
Rana clamitans (Green Frog)	Ant. Pit. Lobe Fresh Homogenate	1	9.0	10.4	Positive
		1/2	25.9		
		1/4	33.3		
		1/8	33.3		
Rana pipiens sphenocephala (Southern Leopard Frog)	Ant. Pit. Lobe Fresh Homogenate	1	28.5	15.8	Positive

TABLE 13 - Continued

Pituitary Source	Nature of Preparation	No. Pit. in 10 ml. Fluid	Percentage Ovulation		General Results
			Heteroplastic	Controls	
Amphibia - Continued					
Microhyla c. carolinensis (Narrow-mouth Toad)	Ant. Pit. Lobe Fresh Homogenate	1	17.3	43.9	Positive
Bufo t. terrestris (Southern Toad)	Ant. Pit. Lobe Fresh Homogenate	1 1/2 1/4 1/8	65.4 70.9 62.3 48.2	45.6	Positive
Hyla gratiosa (Giant Tree Frog)	Ant. Pit. Lobe Fresh Homogenate	1	39.5	41.6	Positive
Hyla c. cinerea (Green Tree Frog)	Ant. Pit. Lobe Fresh Homogenate	1	10.5	14.7	Positive
Desmognathus fuscus auriculatus (South. Dusky Salamander)	Whole Pit. Fresh Homogenate	3 1 1/2	22.2 31.6 37.1	89.2	Positive

TABLE 13 - Continued

Pituitary Source	Nature of Preparation	No. Pit. in 10 ml. Fluid	Percentage Ovulation Heteroplastic	Controls	General Results
Reptilia					
Natrix taxispilota (Brown Water Snake)	Whole Pit.	1	26.9	45.0	Positive
Sceloporus u. undulatus (South. Fence Lizard)	Whole Pit.	2	13.0	43.9	Positive
	Fresh	1	0.0		
	Homogenate	1/2	0.0		
Aves					
Gallus gallus (Chicken)	Whole Pit.	4	12.7	35.0	Positive
	Fresh	3	42.7		
	Homogenate	2	9.8		
		1	4.2		
Mammalia					
Sus scrofa (Hog)	Ant. Pit.	1/2	0.0	32.1	Positive
	Lobe	1/4	4.2		
	Fresh	1/8	8.7		
	Homogenate	1/16	57.9		
		1/32	13.3		
		1/64	8.6		
		1/128	0.0		

TABLE 13 - Continued

Pituitary Source	Nature of Preparation	No. Pit. in 10 ml. Fluid	Percentage Ovulation Heteroplastic	Controls	General Results
Mammalia - Continued					
Sus scrofa (Hog)	Whole Pit. Fresh Homogenate	1/2	0.0	32.1	Positive
		1/4	0.0		
		1/8	2.9		
		1/16	31.8		
		1/32	28.6		
		1/64	13.0		
		1/128	0.0		
		Grams of Pit. in 10 ml. Fluid			
	Whole Pit. Desiccated Powder	0.1	7.1	32.1	Positive
		.025	5.6		
		.006	30.8		
		.00016	15.0		
		.00004	47.8		
		.00002	0.0		
	Ant. Pit. Desiccated Powder	0.1	36.8	32.1	Positive
		.025	20.0		
		.006	20.8		
		.00016	22.2		
		.00004	8.3		
		.00002	0.0		

TABLE 13 - Continued

Pituitary Source	Nature of Preperation	Grams of Pit. in 10 ml. Fl.	Percentage Ovulation Heteroplastic	Controls	General Results
Mammalia - Continued					
Sus scrofa (Hog)	Gonadotropic Fraction Desiccated Powder	0.1	0.0	32.1	Negative
		.025	0.0		
		.006	0.0		
		.00016	0.0		
		.00004	0.0		
		.00002	0.0		
Ovis aries (Sheep)	Whole Pit. Desiccated Powder	0.1	11.5	32.1	Positive
		.025	52.0		
		.006	34.6		
		.00016	17.4		
		.00004	29.4		
		.00002	0.0		
Sus scrofa and Ovis aries	Synergistic Powder 90% Hog and 10% Sheep Ant. Pit.	0.1	0.0	45.0	Positive
		.025	65.4		
		.006	56.7		
		.00016	44.0		
		.00004	11.0		
		.00002	-		
		Amount Preg. Urine Extract in 10 ml. Fluid	Preg. Urine		
Homo sapiens	Pregnancy Urine Extract (Acetone-ether Technique)	Extract from 40 ml. preg. urine	0.0	38.6	Negative

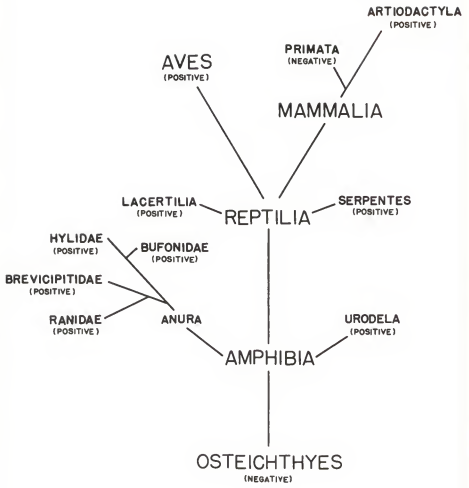

Fig. 8. Diagrammatic phylogenetic tree showing the general effect of various pituitary materials upon in vitro ovulation in Scaphiopus holbrooki.

Physiological and Ecological Relations to the Breeding Stimulus

It is generally recognized by herpetologists and naturalists that, throughout its range, the eastern spadefoot toad (Scaphiopus holbrooki) breeds in temporary ponds during the warmer months of the year, following periods of excessive rainfall. In fact, the principal breeding cue recognized by this species seems to be excessive precipitation, providing the air and ground are not near freezing temperatures.

An excellent insight into the ecological requirements and breeding behavior in the genus Scaphiopus is given by Bragg (1945). He points out that all spadefoots exhibit a xeric pattern of breeding characterized by 1) lack of a definite breeding season, 2) use of temporary water only, 3) breeding behavior in nature initiated only by the coming of rain, and 4) males and females attracted to a chorus by the loud voices of males. Bragg further mentions that the eastern spadefoot seems to be stimulated by large amounts of rainfall, rather than by the violence or rate of fall as is characteristic of S. bombifrons, S. harmondi, and S. couchi. He also points out that low temperatures may inhibit breeding by members of this genus.

Perhaps the best work dealing with the breeding of the eastern spadefoot toad is that of Ball (1936). He states that in order for

breeding to occur, "the ground temperatures must have risen above 2.5° C. in the stratum occupied by the toads, and sufficient rain must have fallen to saturate the soil."

From the literature and through personal communication, breeding records of the spadefoot were obtained from thirty-eight naturalists (Table 14). Records of 121 different breeding choruses from localities over the entire range were included. Data on the type of breeding pond, the time of day at which breeding occurred, precipitation, and temperature were also given when available. Weather data for all Florida breeding choruses were obtained from the records of the Weather Bureau (1921-1955). These records reveal certain significant facts.

Breeding Season

The first record regarding the length and time of the breeding season for the spadefoot was made by Sherwood (1897) who stated that, "the eggs are laid anytime from April to June." Since that time a number of workers have made statements concerning the length of the breeding season. Driver (1936) wrote that the spadefoot does not have a regular season for deposition of ova, but that the eggs may be laid anytime from April 1 to August 31. In 1936, Ball reported that the spadefoots of Connecticut bred as early as April and as late as August. Concerning the spadefoot in Florida, Carr (1940)

TABLE 17

SPADEFOOT TOAD BREEDING RECORDS

Source of Record	Location of Breeding Site	Date of Breeding Chorus	Inches of Rainfall on Breeding Date	Air Temp. on Breeding Date °F.	Water Temp. on Breeding Date °F.	Type of Breeding Site	Time of Breeding Chorus
Allen, J.A. (1868)	Springfield, Mass.	May 27-28, 1866	-	-	-	Transient Pool	-
		June 1863	-	-	-	Temp. Pool	-
	Cambridge, Mass.	April 1, 1868	-	-	-	-	-
		Ap. 15, 1868	-	-	-	-	-
		May 14, 1868	-	-	-	-	-
		May 22, 1868	-	-	-	-	-
Ball, S.C. (1936)	Danvers, Mass.	Aug. 12, 1834	-	-	-	-	-
		June 16, 1842	-	-	-	-	-
	Boston, Mass.	Ap. 19, 1863	-	-	-	-	-
		Ap. 29, 1863	-	-	-	-	-

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Driver, E. C. (1936)	Northampton, Mass.	Ap. 18, 1933	-	-	-	Temp. Rain Pond	Night
		Ap. 17, 1934	-	-	-	Temp. Pool	Night
		Ap. 24, 1934	-	-	50-60	-	Night
Dunn, E. R. (1930)	Northampton, Mass.	May 3, 1928	-	-	-	-	-
Hargitt, C.W. (1888)	Martha's Vineyard, Mass.	Aug. 10, 1887	-	-	-	Temp. Pond	Day
Nichols, A. (1852)	Essex Co., Mass.	July, 1825	3.14	-	-	Temp. Basin	-
		Aug. 12, 1834	-	-	-	Temp. Basin	-
		June 16, 1842	-	-	-	Temp. Basin	Day
Putnam, F.W. (1865)	Cambridge, Mass.	Ap. 19, 1863	-	-	-	-	-
		Ap. 29, 1863	-	-	-	-	-
Wright, A.H. (1932)	Framingham, Mass.	June 16, 1842	-	-	-	-	-

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Ball, S.C. (1936)	Ansonia, Conn.	Ap. 15-18, 1933	1.66	42-54	-	Temp. Pond	Day & Night
		June 11, '33	-	-	-	Temp. Pond	Night
		Ap. 16-17, '34	1.91	45-50	-	Temp. Pond	Day & Night
		May 2-3, '34	-	-	-	Temp. Pond	Day & Night
		June 19, '34	-	-	-	Temp. Pond	Night
		June 18, '35	-	-	-	Temp. Pond	Day & Night
	Westville, Conn.	Ap. 22, '33	-	-	-	-	-
	Mt. Carmel, Conn.	Ap. 19, '34	-	-	-	Temp. Pool	Night
Smith, F.S. (1879)	New Haven, Conn.	April 29, 1879	-	-	-	Rain Pond	Day
Ball, S.C. (1936)	Long Island, N.Y.	July, 1884	-	-	-	-	-
	Jamaica, L.I., N.Y.	July 17, 1885	-	-	-	-	-

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Nichols, J. T. (1917)	Long Is., N.Y.	June 17, 1916	-	-	-	Woodland Pool	Day
		July 23, 1916	-	-	-	Pool in Pasture	Day
Overton, F. (1914)	Patchoque, L.I., N.Y.	April, 1912	-	-	-	Temp. Pool	Day
		Ap. 27, '12	-	-	-	Temp. Pool	-
		Ap. 12-13, '13	-	-	-	Temp. Pool	Night
		Ap. 27-28, '14	-	-	-	Small Perm. Pool	-
		May 5-6, '14	-	-	-	-	Night
		July 7, 1914	-	-	-	Salt Marsh	Night
Overton, F. (1915a.)	Patchoque, L.I., N.Y.	May 22, 1915	-	-	-	Temp. Pool	Night
(1915b.)	Patchoque, Yaphank, Middle Island, Coram L.I., N.Y.	Aug. 4-5, 1915	-	-	-	Numerous Temp. Pools	Night
Pike, N. (1886)	Long Island, N.Y.	Aug. 8, 1884	-	-	-	-	Day & Night

TABLE 14- Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Smith, R. H. (1952)	Albany, N.Y.	Ap., 1940	-	-	-	Cellar Hole	-
		Ap. 9, 1951	-	-	-	Rain Pools	-
Richmond, N.D. (unpubl. data)	Montandon, Penn.	May 26, 1943	-	-	-	Temp. Pool	-
Abbott, C. C. (1884)	Trenton, N.J.	May, 1874	-	-	-	-	-
		Ap. 10, 1884	-	-	-	Sink Hole Pond	-
		Ap. 27-28, 1884	-	-	-	-	-
		June 25-26, 1884	-	-	-	Sink Hole Pond	Day & Night
Davis, W. T. (1908)	Lakehurst, N.J.	Aug. 1-2, 1906	-	-	-	Temp. Pool	-
Simmons, R. S. (unpubl. data)	Massey, Md.	April 1, 1952	-	65	-	Flooded Fields	Night

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Richmond, N. D. (1947)	New Kent Co., Va.	Aug. 15, 1940	-	77-84	78	Large Pools	-
		Mar. 15, 1944	-	-	40-58	Large Pools	-
Richmond, N. D. (unpubl. data)	New Kent Co., Va.	May 26, 1938	-	-	-	Temp. Pool	-
		Aug. 15-16, '40	-	-	-	Temp. Pool	-
		July, 1941	-	-	-	Temp. Pool	-
		Mar. 26-27, '43	-	-	-	Temp. Pool	-
		Mar. 12, 15-17, 1944	-	-	-	Temp. Pool	-
		Mar. 23-24, 27-28, 1944	-	-	-	Temp. Pool	-
		Ap. 23-24, 1944	-	-	-	Temp. Pool	-
		May 27, 1945	-	-	-	Temp. Pool	-
		June 19, 1945	-	-	-	Temp. Pool	-
		July 18, 1945	-	-	-	Temp. Pool	-
		July 2, 1946	-	-	-	Temp. Pool	-
		May 26, 29, '48	-	-	-	Temp. Pool	-
	Radford, Va.	Mar. 26, 1935	-	-	-	Temp. Pool	-
	Ceredo, W. Va.	June 27, 1940	-	-	-	Temp. Pool	-

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Green, N.B. and N.D. Richmond (1940)	Huntington, W. Va.	April 16, 1939	1.67	-	-	Flooded Field	Day & Night
	Wayne Co., W. Va.	April 17, 1939	-	-	-	Temp. Pools	-
Green, N.B. (1948)	Lawrence Co., Ohio	June 2, 1946	1.85	-	-	Temp. Pools in Field	Night
		June 8, 1947	2.58	-	-	-	Night
Spangler, P. J. (1950)	Athens Co., Ohio	June 1, 1950	-	-	-	Temp. Pond	-
		June 16, 1950	-	-	-	Temp. Pond	-
Swanson, P. L. (1938)	Shoals, Indiana	May 3, 1937	-	-	-	Rainwater Pools	-
Giovannoli, L. (1936)	Horse Cave, Ky.	June 3, 1935	-	68	-	Temp. Pond	Night

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Brandt, B. B. (1936)	North Carolina	March 20, 1933	-	60-82	-	Temp. Pool	-
Brimley, C. S. (1896)	Raleigh, N.C.	May, 1895	3.46	-	-	-	-
Obrecht, C. B. (1946)	Horry Co., S.C.	July 11, 1945	-	-	-	Rain Pools	-
Quinby, J. A. (1954)	Dorchester Co., S. C.	March 11, 1953	1.59	-	-	Flooded Bottom Land	Night
Schwartz, A. (unpubl. data)	Dorchester Co., S.C.	April 7, 1954	-	-	-	Temp. Pool	Night
	Kershaw Co., S. C.	June 20, 1952	-	-	-	Rain Pools	Night
	Charleston Co., S.C.	Sept. 16, 1932	-	-	-	Temp. Pool	-
		Feb. 26, 1939	3.5	66	-	Temp. Pools	-
		Feb. 11, 1940	-	-	-	-	Night
		Aug. 12, 1940	-	-	-	-	-

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Schwartz, A. (unpubl. data)	Charleston Co., S.C.	Aug. 20, 1949	-	-	-	-	-
		July 8, 1950	-	-	-	-	Day
		Feb. 7, 1953	-	-	-	Temp. Ditches	Night
Crenshaw, J.W. (unpubl. data)	Baker Co., Georgia	April, 1948	-	-	-	Temp. Pools	-
Simmons, R. S. (unpubl. data)	Statesboro, Georgia	March, 1953	-	-	-	-	-
Wright, A. H. (1932)	Chesser Island, Ga.	June 14, 1922	-	-	-	-	-
Bell, L. N. (unpubl. data)	Miami, Fla.	Oct. 8, 1952	3.45	73	-	-	Day & Night
		Oct. 27, 1952	6.94	74-76	-	-	Day & Night
		Sept. 18-19, 1953	1.66	70-87	-	Rain Pond	-

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Bell, L. N. (unpubl. data)	Miami Beach, Fla.	June 27, 1953	1.39	73-84	-	-	-
	Homestead, Fla.	June 25, 1954	0.56	69-90	-	-	-
Carr, A. F. (1940)	Tarpon Spgs., Fla.	Sept. 1-3, 1935	1.67	74-83	-	Temp. Pools	Day & Night
Deckert, R. T. (1921)	Miami, Fla.	May 16, 1920	3.14	69-76	-	Flooded Streets	Day & Night
Einem, G. E. (unpubl. data)	Melbourne, Fla.	Oct. 9, 1953	3.56	70-76	-	Temp. Pool	Day & Night
Goin, O. and C. J. Goin (unpubl. data)	Gainesville, Florida	Sept. 24, 1947	3.34	62-83	-	Temp. Pond	Night
		Mar. 9, 1948	2.13	58-80	-	Temp. Pond	Night
		Ap. 5, 1949	4.85	62-70	-	Temp. Pool	Day & Night
		Aug. 22, 1949	3.21	71-91	-	Temp. Pool	Night
		Sept. 5, 1950	5.60	73-75	-	Temp. Pool	Day & Night

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Goin, O. and C. J. Goin (unpubl. data)	Gainesville, Florida	Oct. 18, '50	1.85	68-75	-	-	Night
		Sept. 18, '51	2.18	70-85	-	Temp. Pool	Day
		Feb. 16, '52	3.14	61-70	-	Temp. Pool	Night
		Mar. 11, '53	2.51	62-69	-	-	Night
		Ap. 7, 1953	4.58	62-73	-	Temp. Pool	Day & Night
		June 14, '53	3.70	66-85	-	-	Night
		June 25, '53	1.70	72-94	-	Temp. Pool	-
		Aug. 23, '53	2.60	67-84	-	Temp. Pool	Day
Hansen, K. L. (unpubl. data)	DeLand, Florida	Oct. 19, '50	3.65	71-79	-	Temp. Pool	Night
	Gainesville, Florida	Feb. 7, 1955	0.61	50-70	64	Temp. Pond	Night
Ober, D. (unpubl. data)	Lakeland, Fla.	Jan. 16, 1955	0.39	55-75	-	Temp. Pools	Night

TABLE 14 - Continued

Source	Locality	Date	Rainfall	Air Temp.	Water Temp.	Site	Time
Pearson, P.G. (1954)	Micanopy, Fla.	Sept. 22, 1951	1.61	73-94	-	-	-
Van Hyning, T. (1923)	Gainesville, Florida	Mar. 18, 1923	-	-	-	-	-
Wright, A. H. (1932)	Hillard, Florida	Aug. 16-17, 1922	2.01	69-91	75-80	Temp. Ponds	Day
Allen, M. J. (1932)	Harrison Co., Miss.	March, 1931	-	-	-	-	-
Kuenzler, E. J. (unpubl. data)	Biloxi, Miss.	Feb. 6, 1955	1.42	52-68	-	Temp. Pools	Day

wrote that the species breeds "from the summer months to middle October." Wright and Wright (1949) stated that this toad breeds from March to September at periods of heavy rainfall. Finally, Pearson (1954) suggested that the spadefoot does not have a particular breeding season in a cyclic, seasonal sense, but rather, breeds only during or after very heavy rainfall.

The data in Table 14 show that Scaphiopus breeds in each month from January through October. Thus far, breeding choruses have not been reported from the months of November or December, and this is almost certainly due to low temperature. When breeding dates are considered from a geographical standpoint, some striking differences are noted. Figure 9 shows the breeding records, by months, for toads from the northern, central, and southern portions of the range. These data give a good insight into the pattern of breeding over the entire range of the spadefoot but do not necessarily present a completely representative picture of breeding.

The records show that in the northern parts of the range (Mass., Conn., and N.Y.) breeding of toads is limited to a five month period (April to August). The great preponderance of breeding (44 percent) occurs in April, and the curve then drops off sharply to the month of August (Fig. 9). The toads of the middle eastern states (N.J., Va., W. Va., Ohio, Ind., Ky., and N. C.) breed over a six month

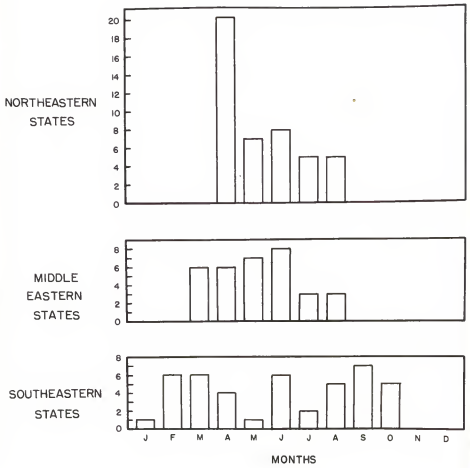

Fig. 9. Monthly breeding records of spadefoot toads from the northern, central, and southern portions of the range.

period, beginning in March and reaching a peak in June. Breeding dates for the southern group (S.C., Ga., Fla., and Miss.) presented a bimodal distribution, with peaks in winter (February) and fall (September).

Breeding in Florida probably occurs in any month of the year, providing the environmental conditions reach an optimum state. The breeding pattern as recorded from twenty-seven choruses presents an interesting case. These records show that occasional breeding occurs during January and February followed by a rise in the spring with the peak in March. There is a decline in May, but it is quite high again in June. For some reason, there are no breeding records for the month of July. A gradual increase in breeding takes place in August, September, and October with the mode for the entire year attained in September. (Fig. 10).

Precipitation

The majority of naturalists state that spadefoot breeding is associated with heavy rainfall. Ball (1936) wrote, "Warm weather alone does not elicit the spring mating response; there must be heavy rain." In 1936, Driver stated, "Apparently the only opportunity for the development of a new generation of spadefoots occurs when more than average rainfall comes in late spring or early summer."

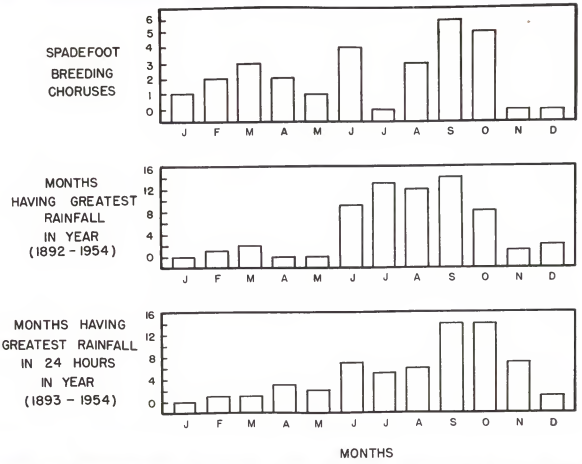

Fig. 10. Correlation of rainfall with spadefoot breeding in the state of Florida.

Goener and Black (1954) reported that, "Scaphiopus holbrooki usually breeds during periods of two to three days, its reproductive activities being in large rain-initiated." When the amounts of precipitation associated with all of the breeding records were averaged, a mean value of 2.69 ± 0.21 inches was obtained. The amount of rainfall associated with breeding ranged from 0.39 to 6.94 inches.

In the state of Florida an interesting relationship exists between rainfall and spadefoot breeding. The fact that, so far as records are concerned, the greatest concentration of breeding in Florida has occurred in the month of September may be correlated with rainfall (Fig. 10). Between the years of 1892 and 1954, the heaviest total precipitation for any single month of the year, occurred most frequently in September, and from 1893 to 1954, the months which had the greatest rainfall in a twenty-four hour period were September and October. A further check of the climatological records at the various breeding localities over Florida, showed that fifteen of the twenty choruses occurred on the day of the month having the greatest precipitation. In their report on the climate of Florida, Mitchell and Ensign (1928) wrote that hurricanes and tropical storms may be expected during the late summer and early fall. Five of the eleven choruses which have been recorded in the late summer and early autumn in Florida took place during or following hurricanes. It therefore appears fairly certain that the August-

September-October increase in breeding is correlated with the hurricanes which come at that time of the year.

Temperature

Precipitation is absolutely essential to breeding by the spadefoot toad, but temperature conditions must also be favorable. Bragg (1945) reports that spadefoot breeding is inhibited by low temperature. He shows that breeding by S. bombifrons usually occurs at temperatures above 52° F., although males may call at a temperature of about 48° F. (This temperature limit is in almost perfect agreement with the temperature limit (50° F.) for in vitro ovulation in the eastern spadefoot.) Earlier, Ball (1936) had asserted that breeding took place only after the temperature of the ground occupied by the toads had risen above 36.5° F. He admitted, however, that no spadefoots bred in 1933 or 1934 until the air temperature had risen to 46° and 49° F., respectively.

The temperature limits just mentioned are in close agreement with some records gathered by the writer near Gainesville, Florida. In January, 1955, the Gainesville Weather Station reported precipitation and minimum-maximum temperatures as follows: January 22) 0.33 inches, 47° - 62° F.; 23) 1.97 inches, 45° - 48° F.; and 24) 0.45 inches, 38° - 41° F. On the nights of January 23 and 24, a thorough

reconnaissance was made of areas known to be inhabited by toad populations, but no breeding was found. Air temperatures of these areas were 40° - 41° F. on both nights. Sufficient rainfall had fallen by the night of January 23 to form numerous temporary ponds in the lower fields, basins, and roadside ditches. However, the low temperatures inhibited breeding on both nights. That temperature was the limiting factor is almost certain, since toads of this area bred on February 7 after less rainfall than on the previous January dates. However, the temperature had risen about 10° F. with a minimum-maximum temperature of 50° - 70° F. The water temperature in one of the breeding ponds was 64.5° F. On March 28 and 29, 1955, a total of 1.28 inches of rain fell. The minimum temperature on these two days was 37° and 33° F., respectively. A trip was made to the same areas which had breeding choruses in February. No breeding choruses were heard. A quick reconnaissance of the fields adjoining the temporary pools showed that no toads had emerged from their burrows. Again, temperature seemed to be the factor limiting breeding. Summarizing these field records, it appears that the lower-limiting temperature for breeding in nature lies between 45° and 50° F.

The average of thirty-four minimum air temperatures for the breeding choruses shown in Table 14 was 65.2 $\pm$ 1.66° F. The mean maximum air temperature was 79.2 $\pm$ 1.86° F. The mean air temperature for the thirty-four choruses was 70.8 $\pm$ 1.48° F. The relatively few

temperature records for the water of breeding ponds gave a mean of 65.5 ± 5.01° F. The extreme mean air temperatures ranged from a low of 47° to a high of 83° F. Water temperatures ranged from 49° to 78° F.

Time of Breeding

It is generally accepted that the eastern spadefoot is exclusively nocturnal in habit. It might then be expected that this species would breed only at night. However, both Gilmore (1924) and A. H. and M. S. Trowbridge (1937) reported diurnal breeding by S. bombifrons. In discussing the factors initiating breeding in Scaphiopus, Bragg (1945) mentioned that heavy rain storms may overcome the tendency toward nocturnal habits. He added, however, that he had found no case of S. holbrooki breeding in daylight. A survey of the records in Table 14 shows, however, that of the fifty-six cases in which the time of breeding was recorded, twenty-six choruses, or forty-six percent, took place at least in part in the daytime. In fifteen of twenty-six cases, the toads either started calling and breeding in the day and continued into the night, or began at night and continued into the day, but in eleven instances, breeding took place exclusively in the day. In general, daytime breeding was probably accompanied by overcast, cloudy skies and rainfall. However, Wright (1932) stated that one breeding occurred in full daylight.

From the total of fifty-six cases, fifty-four percent of the choruses bred exclusively at night.

Type of Breeding Ponds

As has been mentioned, Bragg (1945) stated that spadefoots breed only (or almost exclusively) in temporary water. Both Tanner (1939) and Bragg (1945) postulate the origin of the genus Scaphiopus under xeric conditions in southwestern North America. Presumably this group lived here long enough to develop, through a selective process, the xeric breeding pattern associated with the flash floods of the Southwest. Although the eastern spadefoot now occupies essentially a mesic environment (woodlands, flood plains, etc), it has retained the desert type of breeding and still follows the general pattern of breeding after unusually heavy rainfall.

In this connection, it is of interest to note that in Table 14 there are eighty-three references to breeding ponds, but only one of these was a permanent pond. Overton (1914) reported the occurrence of an April chorus in a small permanent pond, but heavy rains were recorded for the date of this breeding, and the toads moved to this pond only after stimulation by rain. The vast preponderance of records would seem to prove rather conclusively that this toad breeds in water that stands for a limited period.

Multiple Breedings in a Single Year

From the assemblage of data in Table 14, it was found that, if the necessary environmental conditions were fulfilled, a single population would breed more than one time in a given year. Nine different observers recorded this phenomenon. It is possible that a portion of a population breeds on a given date, and another portion breeds at a later time. It is also conceivable that several complements of eggs and sperm are produced in a given year. Ball (1936) was of the opinion that not all of the females in a given locality necessarily oviposited at the first favorable environmental opportunity. Driver (1936) stated, "Possibly some observers have been misled into thinking that only one spawning occurred, when actually the spadefoots may make several emergences during one season."

There is the possibility that the majority of a population might breed on the first favorable occasion, with no subsequent breedings occurring even though optimum environmental conditions prevail. Brandt (1936) described a heavy breeding on March 20, 1933, following four days of intense warm rains. He wrote, "Whether a second congress can be initiated by a recurrence of such conditions remains undetermined; however, a similar combination of conditions of heavy rainfall and high temperature failed to bring about a congress in May of the same year."

The following are the annotated records of known populations which bred more than one time in a given year. Allen (1868) reported the breeding of one population on four different occasions during a single year at Cambridge, Massachusetts. In 1934 Driver (1936) recorded a double breeding in the month of April (17 and 24) at Northampton, Massachusetts. At Cambridge, Massachusetts, Putnam (1865) listed two choruses breeding in the same pond on April 19 and April 29, 1863. Ball (1936) recorded the breeding of a population of spadefoots at Ansonia, Connecticut on April 15-18 and again on June 11, 1933. In 1934, the same population bred on three different occasions (April 16-17, May 2-3, and June 19). Overton (1914) gave three dates (April 27-28, May 5-6, and July 7) for the same population at Patchoque, L.I., New York in 1886. Abbott (1884) recorded breeding on April 10, April 27-28, and June 25-26 in 1884 in Trenton, New Jersey. Richmond (personal communication) listed a series of five breedings for one population near Lanexa, Virginia during 1944. Spadefoots bred on March 12, 15-17, 23-24, 27-28, and on April 23-24. In 1945, the same population bred on May 27, June 19, and July 18. A double breeding occurred in May of 1948, on May 26 and again on May 29. The records by Dr. and Mrs. C. J. Goin, in Gainesville, Florida show that breeding choruses were formed on April 5 and April 22 in 1949. In the unusually wet year of 1953 they noted five separate breedings, occurring on March 11, April 7,

June 14, June 25, and August 22. Pearson (1954) mentioned that one population near Gainesville bred on April 7 and August 23 of 1943.

Extended Periods of Time without Breeding

That environmental conditions are necessary to breeding is evidenced by the fact that populations in a given locality are known to have gone extended periods without breeding. Commenting upon the infrequency of their breeding, Ball (1936) wrote, "Remarkable also is the apparent irregularity of its emergence in numbers for breeding. In the North during the past 125 years, only about sixteen instances have been recorded, all associated with heavy rains." From this statement, it might be inferred that the spadefoot does not breed every year. However, Ball believed that the adult females bred every year provided that environmental conditions were suitable.

The records now available prove that some populations do skip one or more years in breeding. It is almost certain that this is due to the lack of heavy rainfall, optimum temperatures, or both. The records which demonstrate this phenomenon are given below. Ball (1936) reported that spadefoots appeared in Danvers, Massachusetts in the summers of 1812, 1825, and 1834, and were not noticed between those years. Abbott (1884) observed a population breeding in Trenton, New Jersey in 1874 and again in 1884. Simmons (unpublished data) wrote that he had personally checked on a known population in

Maryland following every rain, and that it had not bred for three years. Richmond (1947) described a chorus on August 15, 1940, at a certain locality in New Kent County, Virginia. He mentioned that this population had not laid in the previous year. In a letter to the writer, Richmond stated that this same population failed to breed during the years of 1942, 1952, and 1954. During the exceptionally dry year of 1954, the writer observed no breeding choruses in the area around Gainesville, Florida. The last breeding record by Dr. and Mrs. Goin of Gainesville, was on August 23, 1953. The population near their home has not bred since that time, a span of twenty-three months.

Experimental Procedures and Results Having Ecological Implications

The majority of frogs and toads seem to follow a rhythmic, seasonal breeding pattern. In many species, the seasonal rise in air and water temperature seems to initiate breeding. The gonads of these forms, which undergo a seasonal hypertrophy, are full and mature in the spring following the winter hibernation. Wright (1946) suggests that FSH (Follicle-stimulating Hormone), secreted by the pituitary during hibernation, stimulates follicular growth, ovarian maturation, and a progressive increase in responsiveness from fall to spring. Undoubtedly, a full complement of sperm is also brought to maturation under the inducement of the gonadotropins. It is

possible that the cyclic, seasonal maturation of the gonads as well as the breeding response has become a genetically fixed physiological function and behavior pattern, or instinct (i.e., "a complicated reaction that an animal gives when it reacts as a whole, and as a representative of a species rather than as an individual, which is not improved by experience, and has an end or purpose of which the animal cannot be aware." - Wheeler, 1926).

When the breeding pattern of the spadefoot toad is considered, an entirely different picture is seen. The xeric type of breeding (Bragg, 1945) is not limited to a seasonal, cyclic pattern. Rather, it tends to be non-seasonal and may be initiated at any time of the year when proper climatic conditions prevail. This would suggest that the stimulus is not behavioral, but is wholly contingent upon climatic conditions. From the preceding review of breeding records (Table 14), only two climatic conditions which influence ovulation are evident; namely, heavy rainfall (sufficient to form temporary ponds) and temperature above 45° - 50° F. The question arises as to how these two environmental factors stimulate the physiology and behavior of the toad so as to initiate breeding and whether there are other environmental factors which may also play a role in this process. By means of the in vitro ovulation technique, it was hoped that the exact method of stimulation might be determined.

It is rather generally accepted that the onset of the breeding response in anurans is hormonally induced. Very recently, Houssay (1954) admitted that the mechanism by which the nervous system acts upon the pars distalis is still unknown. It is believed that this mechanism involves neural stimulation. It is quite probable that the environmental conditions of optimum temperature and heavy rainfall in some way act upon the toad's nervous system, which in turn stimulates the endocrine system. Noble (1931) stated that ovulation in amphibia is due to the action of hormones from the anterior lobe of the hypophysis, which is under the control of the nervous system. He further suggested that this neural-hormonal response accounts for the close correlation between breeding and favorable climatic conditions.

The extreme sensitiveness of the amphibian ovary to hormones from the pars distalis has made it possible to determine the exact gonadotropins which stimulate and sensitize the ovary. Wright and Hisaw (1946) were able to demonstrate that the FSH principle (fresh sheep pituitary) maintains the frog ovary in a highly sensitized state and that only through the combined action of FSH and LH (Luteinizing Hormone) could ovulation be induced in hypophysectomized frogs. That the amphibian ovary is extremely susceptible to pituitary secretions has been ably demonstrated. Rugh (1939) found that the frog's ovary, following hypophysectomy, would respond with a greater

amount of ovulation *in vivo*: Wright (1946) found this also true of ovulation *in vitro*. Wright was able to show that this increase in ovulation was due to a sensitization of the ovary by gonadotropins released into the blood at the time of hypophysectomy. In 1945, Wright reported that the injection of a subminimal dose of pituitary substance into an intact frog brought about a sensitization of the ovarian tissues and that the response to pituitary factors *in vitro* was greater in sensitized than in untreated ovaries.

It seemed possible, therefore, that if female spadefoots were subjected to the proper stimuli, the ovaries might become sensitized through hypersecretions of gonadotropins. This sensitization could be demonstrated by use of the *in vitro* ovulation technique. It might then be assumed that stimuli which sensitized the ovaries under experimental conditions might also be the stimuli which are important in initiating breeding under natural conditions. To test this hypothesis, female toads were exposed to such conditions as light, abrupt changes of temperature, lowered atmospheric pressure, and water.

Effects of Light and Darkness on Female Toads

It is well known that the secretion of gonadotropins by the *pars distalis* stimulates the breeding response, ovulation, and spermiation in the majority of vertebrates. Since light initiates

this process in some birds and mammals, it seemed logical to test the effects of light on Scaphiopus. However, since this animal is known to be both nocturnal and fossorial, the possibility of positive results seemed somewhat remote.

Fifteen females were placed in absolute darkness immediately after capture. These were measured (head width and weight) in a photographic darkroom under a Stratton Safelight (Series OA), and eight individuals of similar size were selected for the experiment. Four control toads were kept in the darkroom in complete darkness. The four experimental animals were removed to an adjoining room of the same temperature (81° F.) and exposed to continuous light for forty-eight hours. Two 100 watt bulbs were used as the source of light, and one bulb was placed 12 inches from each side of the glass container holding the toads.

At the end of a forty-eight hour period, the control toads were sacrificed in darkness, while the experimental toads were sacrificed in the light, and four pieces of ovary were selected from each toad. Each piece was placed in a vial containing the equivalent of one-half pituitary in 5 ml. of Holtfreter's solution. The individual and mean ovulation percentages are shown in Table 15. The sixteen tests performed on females exposed to light gave a mean of 32.2 $\pm$ 5.5, on those exposed to dark, 26.6 $\pm$ 4.0. When these data were compared

TABLE 15

IN VITRO OVULATION RESULTS OBTAINED FROM FEMALES EXPOSED TO LIGHT AND DARKNESS

Type of Exposure	Number of Toad	Percent Ovulation using 1/2 Pit. in 5 ml. of Holtfreter's Fluid				Mean
Dark	1.	25.0	33.3	14.3	20.8	23.35
	2.	18.7	35.0	31.6	68.0	38.33
	3.	38.7	34.4	9.1	37.5	30.02
	4.	44.0	13.0	0.0	3.0	15.08
						Mean 26.56 ± 3.97
Light	1.	35.0	15.0	16.3	27.3	23.40
	2.	22.2	69.7	41.0	20.2	38.27
	3.	46.2	31.0	50.0	22.7	37.47
	4.	14.3	88.9	12.8	0.0	29.00
						Mean 32.19 ± 5.49

statistically, no significant difference was found to exist ($t. = 0.81$, $P. > 0.05$). From these results it was concluded that light does not act as a stimulus to the toad pituitary and consequently sensitization of the ovaries does not occur.

Effect of Abrupt Changes of Temperature on Female Toads

That temperature has effects upon the breeding response of amphibia is well illustrated by the Spanish newt (Pleurodeles waltl). Noble (1931) reported that females of this species may be induced to ovulate merely by placing them in a low temperature over night and then removing them to a tank suitable for breeding. According to Noble, the sudden rise in temperature initiates the ovulation cycle and both courtship and breeding will frequently follow.

In many instances of spadefoot breeding, the ground temperatures have been quite low and are subsequently raised with the coming of warm rains. Ball (1936) wrote that many of the April breedings in Connecticut come with the first spring rains which thaw and warm the ground. Pearson (1954) reported four breeding choruses in Florida which were associated with cold fronts eventually broken by heavy rainfall. Simmons (personal communication) wrote that a Maryland breeding was preceeded by a week of freezing weather. This cold period was broken by warm rains which evoked the breeding chorus.

In each case, the existing temperature was low and was followed by a rather sudden rise in temperature. In order to test the effects of temperature upon the toads, these conditions were duplicated, as nearly as possible, in the laboratory.

Eight female toads of approximately the same size were used. Four experimental animals were placed in a gallon jar containing one-fourth inch of water and put into a refrigerator set at 54° F. Four control toads were placed in a jar containing water and kept at room temperature (73° F.). After a twelve-hour period, the experimental toads were brought into room temperature and kept there for six hours. Then, both the control and experimental animals were sacrificed; their ovaries were removed, and four fragments of ovary were obtained from each toad. Each fragment was placed in a vial containing the equivalent of one-half pituitary in 5 ml. of solution.

The ovulation results for these tests are shown in Table 16. The mean for the experimental females exposed to lower temperature was 26.3 $\pm$ 3.4 percent and for the control toads held at room temperature, 31.0 $\pm$ 4.0 percent. It was found that no statistical difference existed between these means ($t. = 0.91$, $P. > 0.05$). It was concluded, therefore, that lowered temperature had not sensitized the ovaries.

TABLE 16

IN VITRO OVULATION PERCENTAGES OBTAINED FROM FEMALE TOADS EXPOSED TO ABRUPT CHANGES OF TEMPERATURE

Exposure Temperature	Number of Toad	Percent Ovulation using 1/2 Pit. in 5 ml. of Holtfreter's Fluid				Mean
Toads Exposed to 54° F.	1.	50.0	37.5	27.8	35.7	27.55
	2.	38.7	28.0	2.8	40.7	37.75
	3.	25.6	8.3	39.4	38.3	27.90
	4.	18.4	9.7	16.1	6.2	12.60
					Mean 26.25 ±	3.40
Toads Maintained at 73° F.	1.	33.5	36.9	67.6	22.0	40.00
	2.	22.9	4.3	14.2	16.8	14.55
	3.	44.2	13.3	29.8	30.3	29.40
	4.	34.6	38.0	50.6	42.6	41.45
					Mean 30.95 ±	3.98

Exposure of Female Toads to Lowered Atmospheric Pressure

It is rather well established that spadefoot toads breed during heavy rain storms, extended periods of rainfall accompanied by stationary or permanent cold fronts, and hurricanes. Low atmospheric pressure often accompanies these climatic disturbances. A number of naturalists have expressed their belief to the writer that spadefoot breeding might be stimulated by heavy rainfall and low atmospheric pressure. In a study of the response of salientia to chorionic gonadotropin, Knepton (1951) subjected male spadefoots to a lowered pressure of 726 mm. of mercury (94.2 percent of an atmosphere). He hypothesized that spermiation might be stimulated by lowered pressure combined with an injection of hormone. His results were contradictory, however, since toads exposed to low pressure reacted both positively and negatively (by spermiation) following injections of chorionic gonadotropin.

Therefore, a study of the effects of lowered pressure upon the pituitary-gonad balance of the female seemed warranted. A large pyrex demijohn (9650 ml.) was fitted with a sealed rubber stopper having two glass-tube outlets. One of the glass tubes was bent into a "U"-shaped form which held a column of mercury. This was backed by millimeter graph paper so that fluctuations between levels of the two columns could be read accurately to the nearest

millimeter. The second glass tube was connected to a short length of rubber tubing which served as an exhaust outlet. This was attached to a faucet aspirator and air was evacuated from the bottle to obtain the desired reduced pressure. Double clamps were placed on the rubber hose near the glass outlet. No leakage was evident after a preliminary trial of one week.

On September 17, 1928, a hurricane passed through Gainesville, Florida producing an atmospheric pressure of 28.94 inches (735 mm.) of mercury. This pressure was chosen as the low value for this experimental work. To obtain this pressure, a reading was taken from a local barometer, and the difference in millimeters of mercury between the current atmospheric pressure and the desired pressure was determined. Air was then exhausted from the bottle until the desired pressure was obtained. Control toads were kept at the atmospheric pressure of the room. Minor pressure fluctuations, due to changing weather conditions, over the forty-eight hour period of the experiment, affected both experimental and control animals alike. It was recognized that a synergistic effect of lowered pressure and water might stimulate the toads to increased ovulation. Therefore, two experiments were conducted. In the first, the experimental and control animals were put into jars containing no water, while, in the second, both experimental and control toads were kept in one-fourth inch of water.

The ovulation results from both experiments are recorded in Table 17. No statistical differences were found between the means of the experimental and control females kept in a dry state ($t. = 0.59$, $P. > 0.05$). Likewise, females exposed to normal and low temperatures while seated in water gave no significant differences in ovulation results ($t. = 1.30$, $P. > 0.05$). It was concluded, therefore, that lowered atmospheric pressure had no effect upon ovarian sensitization.

It was of interest to note that those animals placed in water had a higher mean ovulation percentage than the toads kept in the dry condition. When the ovulation means for the wet and dry experimental toads were compared, a significant difference was found to exist ($t. = 2.99$, $P. < 0.01$). However, the comparison between control animals exposed to wet and dry conditions failed to attain significance ($t. = 1.28$, $P. > 0.05$). In the following section, consideration is given the role that water plays in the stimulus of the toad's pituitary gland.

Effects of Water Upon Female Toads

Rather early in the study it was found that female toads brought directly from the field occasionally gave low or negative ovulation results. It was noted that this peculiarity seemed to be correlated

TABLE 17

IN VITRO OVULATION RESULTS FROM TOADS EXPOSED TO LOWERED ATMOSPHERIC PRESSURE

Water Relation	Number of Toad	Control Atm. Pressure - Approx. 760 mm. of Mercury			Experimental Atm. Pressure - Approx. 735 mm. of Mercury		
		Percent Ovulation From Following Pit. Dilutions in 10 Ml. Fluid					
		1/2	1/4	1/8	1/2	1/4	1/8
Females in Water	1.	36.2	16.7	3.2	47.1	40.0	16.7
	2.	39.3	24.6	5.8	40.5	34.7	36.4
	3.	13.5	4.2	4.5	4.7	4.7	1.1
	4.	18.2	8.3	4.5	15.4	13.1	12.5
		Mean - 14.15 ± 3.60			Mean - 22.10 ± 4.62		
Females Kept Dry	1.	10.9	7.3	1.1	18.2	7.3	0.0
	2.	2.1	5.0	0.0	10.0	9.5	3.2
	3.	25.7	15.0	2.4	7.5	4.5	0.0
	4.	14.0	6.9	12.7	15.9	3.0	2.1
		Mean - 8.54 ± 2.17			Mean - 6.87 ± 1.59		

with dry soils at the collection site. A few toads kept on moist paper towels in storage jars gave similar results. However, those females held in gallon storage jars containing one-fourth inch of water reacted normally to the in vitro tests.

Preliminary tests showed that those females kept in water, previous to the removal of their ovaries, yielded higher percentages of ovulation, while toads kept under dry conditions gave very low ovulation results. Occasionally, big females retaining large amounts of fluid in their bladders and lymph sacs gave normal ovulation results in spite of subjection to dry conditions. In general, however, there seemed to be a direct correlation between percentage of ovulation in vitro and exposure to water. Three different types of experiments were performed to investigate this relationship of water to ovulation.

Exposure of Females to Wet and Dry Conditions. In the first experiment, experimental toads were kept in finger bowls containing 100 ml. of tap water, and control toads of similar size were placed in bowls which were dry. Urine was removed from the dry containers throughout the period of exposure. The animals were held under these conditions for a period of forty-eight hours and then sacrificed for their ovaries. These were exposed to stock pituitary dilutions and the ovulation percentages obtained. A total of fifteen comparisons

of this type were conducted using thirty females and thirty males (pituitary donors). The individual and mean ovulation percentages are shown in Table 18, while a graphic comparison of these results is shown in Figure 11.

A statistical analysis was made between the ovulation percentages, at the one-half pituitary level, for toads exposed to wet and dry conditions (Table 19). A significant difference was found to exist in this comparison ($t. = 2.95$, $P. < 0.05$). A second more generalized analysis was made in which ovulation percentages were taken from all pituitary dilutions (one-half to one-sixteenth) under the two conditions and compared (Table 19). Again, a significant difference was obtained, with $t. = 4.4$, and $P. < 0.001$. It is rather generally known that anurans demonstrate active water-uptake until a physiological optimum is reached, and, from these results, it appears that the presence of water is necessary to normal ovulation.

Exposure of Females to a Soil-Moisture Gradient. After the water-ovulation relationship had been demonstrated, it seemed worthwhile to investigate the amount of moisture needed to induce a physiologically optimum state for normal ovulation. The spadefoot lives in a burrow and is undoubtedly affected by fluctuations in soil moisture. Therefore, an experiment was designed using a gradient of soil moistures. It was hoped that a critical moisture

TABLE 18

IN VITRO OVULATION PERCENTAGES OBTAINED USING OVARIES TAKEN FROM FEMALES SUBJECTED TO WET AND DRY CONDITIONS FOR 48 HOURS

Date	Wet Condition				Dry Condition			
	Amount of Pituitary in 10 Ml. Fluid							
	1	1/2	1/4	1/8	1	1/2	1/4	1/8
6-26-54	46.2	32.5	4.0	15.6	2.0	2.9	21.4	4.8
	38.1	38.8	64.1	0.0	0.0	0.0	2.6	0.0
6-28-54	32.9	45.1	18.0	8.7	6.7	0.0	0.0	0.0
	2.3	13.2	3.9	0.0	5.9	12.5	2.9	0.0
6-30-54	7.0	20.8	19.5	34.0	27.5	2.1	10.0	0.0
	49.0	12.8	3.6	0.0	17.6	1.7	1.8	0.0
7-1-54	31.9	13.0	30.8	11.8	5.7	19.2	3.0	0.0
	17.0	30.4	5.9	0.0	17.9	8.8	0.0	0.0
7-6-54	62.5	36.4	42.9	0.0	0.0	0.0	0.0	0.0
	10.0	14.0	9.4	0.0	0.0	0.0	0.0	0.0
7-12-54	12.2	0.0	0.0	2.9	0.0	0.0	0.0	0.0
	11.1	42.2	9.4	5.1	12.8	40.0	9.4	2.6
	25.9	15.7	3.1	0.0	25.0	28.6	30.7	19.0
12-1-54	14.0	-	-	-	0.0	-	-	-
	7.5	-	-	-	0.0	-	-	-
Mean	24.8	24.2	16.5	6.0	8.3	8.9	6.3	2.0

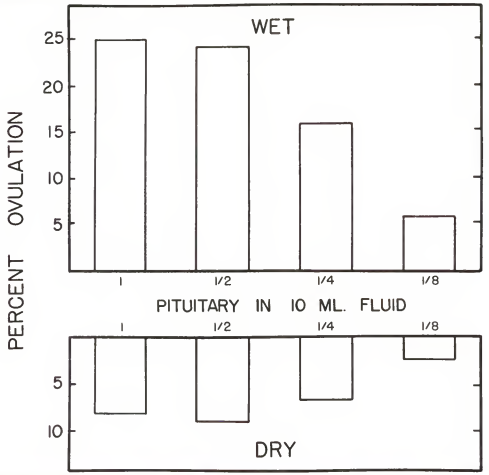

Fig. 11. In vitro ovulation results obtained from female toads exposed to wet and dry conditions.

TABLE 19

TESTS FOR STATISTICALLY SIGNIFICANT DIFFERENCES
BETWEEN PERCENT OVULATION AND MOISTURE RELATIONSHIPS

Pituitary Dilutions	Relation to Water	Mean Percentage Ovulation and Standard Error	Standard Deviation	Number	Significance
One-half Pituitary Suspended in 10 Ml. of Holtfreter's Solution	Females in Water	24.85 ± 4.57	17.70	15	t. = 2.95
	Females Kept Dry	8.34 ± 2.88	11.16	15	P. < 0.01
One-half to one-sixteenth Pituitary in 10 Ml. Holtfreter's Solution	Females in Water	18.50 ± 2.28	16.73	54	t. = 4.43
	Females Kept Dry	6.85 ± 0.17	9.38	54	P. < 0.001

percentage might be found, above which ovulation would proceed normally, but below which water-uptake would be insufficient for ovulation to occur normally.

A large quantity of soil taken from a burrow site was oven dried for forty-eight hours. Soil aliquots of 300 grams were placed in six finger bowls. By dry weight of soil, the following percentages of tap water were added to the six soil samples (50%, 45%, 33%, 25%, 10%, and 0%). In the bowls containing forty-five and fifty percent water, the soil was beyond the point of saturation and a film of water stood above the soil's surface. Each finger bowl was covered by a second inverted bowl to prevent moisture loss. Two female toads were put into each soil sample and allowed to burrow. The toads were removed in forty-eight hours, sacrificed, and their ovaries used in ovulation tests. Four pieces of ovary were taken from each toad and each was suspended in a vial containing 5 ml. of pituitary suspension. Toads exposed to soil moistures of 50, 45, 33, 25, 10, and 0 percent gave mean ovulation percentages of 70.0, 49.0, 48.2, 24.7, 2.7, and 6.3, respectively. Although no sharp break in ovulation occurred at any level of soil moisture, reduction of soil moisture seemed to have a gradual inhibitory effect. This furnishes additional support for the idea that ovulation seems to be correlated with exposure to moisture.

Exposure of Females to Different Humidities. As a final test of the effect of moisture, a series of chambers were employed in which humidities ranged from 0 to 98 percent. These chambers consisted of gallon jars containing an excess of certain salts in saturated solutions. It is well known that a saturated aqueous solution (water) in contact with an excess of a definite solid phase (a salt), will maintain a constant humidity when kept in an enclosed space at a given temperature. From the International Critical Tables (1926), certain salts (Table 20) were selected which would produce desired humidities at 20° C. Excess amounts of these salts were placed in 100 ml. of distilled water in each of five jars of one gallon capacity. To achieve a zero humidity, 150 grams of $CaCl_2$ were placed in a sixth jar. A tightly fitted platform of one-fourth inch hardware cloth was supported 3 inches above the solution by a glassware foundation.

The series of jars were kept in a refrigerator set at 20° C. and animals were given a twenty-four hour period of exposure. Toads held at lower humidities tended to desiccate rapidly. It was for this reason that the period of treatment was reduced from the forty-eight hours employed in the experiments on temperature, moisture, atmospheric pressure, etc. to twenty-four hours. Upon removal from the humidity chambers, toads were reweighed and then sacrificed. Four pieces of ovaries were taken from each toad and suspended in

TABLE 20

IN VITRO OVULATION RESULTS FOLLOWING EXPOSURE OF FEMALE TOADS TO DIFFERENT HUMIDITIES

Excess Salt in Solution	Percent Humidity at 20° C.	Number of Female Toads Used	Number of Ind. Tests Performed on Ovaries of Exposed Females	Mean Percent Ovulation
$Pb(NO_3)_2$	98%	5	20	12.5%
$NaClO_3$	75	5	20	14.1
KSCN	47	4	16	4.7
$KC_2H_3O_2$	20	4	16	2.1
LiCl	15	4	16	3.8
$CaCl_2$ (no water)	0	4	16	1.1

dilutions of pituitary varying from one-half to one-sixteenth anterior lobe in 10 ml. of Holtfreter's fluid.

Mean percentages of ovulation for the gradient of humidities were as follows: 98%) - 12.5; 75%) - 14.1; 47%) - 4.7; 20%) - 2.1; 15%) - 3.8; and 0%) - 1.1. On inspection, this seems to indicate a direct correlation between percentage of ovulation and humidity (Table 20), and this conclusion was validated when the data were analyzed statistically. A strong positive correlation was found to exist between ovulation and humidity ($r = 0.45 \pm 0.16$), and it was found to be statistically significant ($t. = 2.5$, $p. = 0.02$). Therefore, each of the three entirely different types of experiments dealing with moisture indicates that moisture plays a very important role in producing ovulation.

Exposure of Hypophysectomized Toads To Wet and Dry Conditions

Wright (1946) reported that a marked increase in ovulation in vitro followed hypophysectomy, in Rana pipiens, due to the sensitization of the ovary by the release of gonadotropins. Because it had been demonstrated repeatedly in the present work that exposure of females to moisture might bring about ovarian sensitization in the spadefoot, it was decided to test the effects of wet and dry conditions on hypophysectomized animals.

In removing the pars distalis, females were put under a light anesthesia with ether. It was possible to open the gape of the mouth sufficiently so that it was not necessary to cut and extend the angle of the jaw. A small, three-sided flap was cut in the skin of the roof of the mouth. This was folded back posteriorly and the parasphenoid bone exposed. A small hole was drilled through this bone at a medial point intersected by a line drawn through the anterior edges of the two lateral projections. A high-speed, flexible-shaft hand-drill was used along with a dental bur (No. 4 straight handpiece). Upon penetrating the brain case, any excess blood and body fluids were removed by aspiration, but little bleeding occurred throughout the entire operation. The pars distalis was lifted out with a pair of fine-pointed forceps. Care was taken not to remove the pars nervosa and pars intermedia, because of the importance of the former in maintaining water balance. No attempt was made to suture the oral mucosa since it had been reported that the thread and knots only cause additional irritation (Wright and Macintyre, 1950). The animals recovered rapidly from the effects of the anesthesia and the operation.

The pars distalis was removed from four toads in the manner described. Two of these toads were placed in a jar containing one-fourth inch of water. The remaining two toads were held in a dry jar. After a forty-eight hour exposure period, the toads were

sacrificed and their ovaries removed. Four pieces of ovary were taken from each toad and exposed to a pituitary homogenate diluted to the equivalent of one-half pituitary in 5 ml. of Holtfreter's solution.

The ovulation results from the two sixteen-vial series were remarkably similar. The mean percentage of ovulation for hypophysectomized toads exposed to wet and dry conditions was 21.3 $\pm$ 7.1 and 25.6 $\pm$ 6.5, respectively. The means were compared statistically, but were found to lack a significant difference ($t. = 0.40$, $P. > 0.05$). Mean ovulation values for the control toads were 28.8 $\pm$ 7.7 percent for the two toads exposed to water, and 7.3 $\pm$ 1.6 percent for the pair of females subjected to a dry state. A statistically significant difference was found when these two means were compared ($t. = 2.56$, $P. < 0.05$). The individual and mean ovulation results for both the hypophysectomized and normal toads are shown in Table 21.

These results indicate that exposure of hypophysectomized females to varying conditions of moisture produces no significant differences in the percentage of ovulation. Since the percentage of ovulation in all of the hypophysectomized females was approximately the same as that found in normal animals exposed to water, the ovaries of hypophysectomized animals must have been pre-sensitized, thereby eliminating the necessity for water. Seemingly, the ovaries

TABLE 21

IN VITRO OVULATION RESULTS OBTAINED FROM HYPOPHYSECTOMIZED AND NORMAL TOADS AFTER SUBJECTION TO WET AND DRY CONDITIONS

Condition of Females	Exposure Conditions	Percent Ovulation Using One-Half Pituitary in 10 ml. Fluid				Mean
Pars distalis Removed	Dry	16.9	28.5	40.0	52.6	25.6 ± 6.5
		48.6	7.7	5.3	3.8	
	Wet	56.1	54.8	19.0	11.1	21.3 ± 7.1
		12.2	8.6	9.1	5.0	
Normal	Dry	17.9	5.9	10.3	7.7	7.3 ± 1.6
		6.1	2.4	0.0	6.9	
	Wet	16.7	14.7	10.3	18.8	28.8 ± 7.7
		74.4	27.3	58.3	12.9	

of both groups of hypophysectomized animals had been maximally sensitized, since water had no added stimulatory effect. Thus, in Scaphiopus holbrooki, as in Rana pipiens, hypophysectomy seems to bring about a sensitization of the ovary. This may well be, as Wright (1946) indicated, the result of the release of gonadotropins at the time of hypophysectomy, although no attempt was made to determine this in the spadefoot. This leaves unsolved the problem of how exposure of the female spadefoot to water sensitizes the ovary.

Monthly Condition of Spadefoot Gonads in Relation to Breeding Sensitivity

It is well known that certain amphibia resorb their eggs and sperm if favorable environmental conditions do not prevail during their usual breeding season. Rugh, (1951) in discussing Rana pipiens, reports that if the female is forced to retain her eggs beyond the normal breeding period, the ova will start to cytolyze in the ovary. However, field data indicate that the spadefoot, unlike Rana pipiens, is not a seasonal breeder, but may breed in any month of the year when environmental conditions are suitable. Writing about the eastern spadefoot toad, Ball (1936) said, "Scaphiopus seems able to retain its ova and sperm until temperature and water conditions are favorable - even until midsummer." Trowbridge

and Trowbridge (1937), working with S. bombifrons state, "It remains to be shown how long spadefoot females can retain ripe eggs and have them fertilizable when laid." In an attempt to determine how long mature viable sperm and ova might be retained, the condition of the gonads was checked each month for a period of one year.

Monthly Ratio of Ovary Weight to Body Weight. It seemed logical that if the maturation of ovaries (due to an increase in number of eggs, an increase in stored yolk materials, or cell growth) were seasonal, it might be revealed by comparative ratios of ovaries to body weights. Each month, therefore, the ovary weights of ten or more female toads were recorded and averaged. These ratio percentages, along with the number of females sacrificed are shown in Table 22. The means for December and April showed the widest range, but a statistical comparison between the two failed to show a significant difference ($t. = 1.2$, $P. > 0.05$). From these results, it was concluded that in the absence of ovulation, female spadefoots carry a full complement of mature, viable eggs at all times of the year.

Comparative Monthly Study of In Vitro Ovulation. In 1937, Rugh showed that there was a marked seasonal variation in the susceptibility of the ovary of R. pipiens to pituitary-induced ovulation and that a greater amount of pituitary was required to

TABLE 22

COMPARATIVE MONTHLY RATIO OF OVARY WEIGHT TO BODY WEIGHT

Month	Number of Individuals	Mean Percentage Ovary: Body Weight
August, 1954	12	14.8
September, 1954	18	15.9
October, 1954	31	15.7
November, 1954	18	16.5
December, 1954	13	14.0
January, 1955	15	15.5
February, 1955	25	14.7
March, 1955	17	15.7
April, 1955	13	17.2
May, 1955	20	16.5
June, 1955	11	15.3
July, 1955	12	16.6
Total Number	205	Mean 15.7

produce a given amount of ovulation in October than in March. Wright (1945) conducted an *in vitro* ovulation study with this same species and found that there was a progressive increase in response to pituitary materials from November (9.5%) to April (89.0%). Since the spadefoot is known to breed in practically any month of the year, it seemed important to test whether such a seasonal peak of ovulation occurred in this form.

To determine this, a large standardized pituitary solution was needed, so that equal aliquots (held in a frozen state) could be used for monthly *in vitro* tests. To secure sufficient pituitary for such a stock solution presented a problem because of the impracticability of collecting a large number of toads at one time. For this reason, a stock solution, consisting of twelve macerated male pituitaries suspended in 120 ml. of Holtfreter's solution, was prepared each month. This solution was then divided into twelve vials and frozen for future use. Wright (1945) found that frozen pituitary solutions retained their hormone titer for extended periods of time.

Each month one female toad was sacrificed as an ovary donor. Vials of frozen pituitary from each monthly pituitary preparation were thawed, and diluted to the equivalent of one-half, one-fourth, and one-eighth of a pituitary in 10 ml. of fluid. This experiment was begun in August, 1954, and in each consecutive month an additional

vial of pituitary was used, so that by the twelfth month thirty-six vials were employed to test the representative female of the month.

The monthly ovulation results for each frozen pituitary preparation are shown in Table 23. When the monthly ovulation percentages are averaged and compared, the results are found to be fairly consistent, indicating that no seasonality in gonad activity exists. A statistical comparison was made for the ovulation results obtained from the three dilutions used. At the one-half pituitary concentration, the months of February and June had the greatest difference in ovulation means, but when compared statistically these showed no significant differences ($t. = 1.9$, $P. > 0.05$). The months of March and June had the widest divergence in ovulation results in the one-fourth pituitary dilution range; nevertheless, no statistical differences existed ($t. = 1.8$, $P. > 0.05$). The greatest range in ovulation percentages for the one-eighth pituitary dilution were found in March and June, but a statistical comparison showed that the differences were insignificant ($t. = 0.33$, $P. > 0.05$).

Since no peaks in ovary sensitivity were found for any month or season of the year, it may be assumed that the female is capable of breeding during any month. The three statistical comparisons verify this assumption.

TABLE 23

MONTHLY SENSITIVITY OF FEMALE TOADS TO IN VITRO OVULATION

Monthly Frozen Pit. Source	Pituitary Dilutions in 10 ml. Fluid	Percentage Ovulation for Various Pituitary Dilutions											
		Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July
	1/2	36.1	33.2	33.3	42.4	52.9	30.9	45.0	22.7	22.5	19.2	41.3	16.4
Aug.	1/4	21.6	18.6	21.5	27.5	19.4	25.2	27.6	17.5	17.5	12.0	33.3	9.4
	1/8	8.3	8.7	15.4	12.4	4.9	15.4	14.9	5.0	5.0	2.2	16.7	4.2
	1/2		23.4	28.0	22.0	36.0	23.8	38.6	38.4	29.4	45.2	27.8	58.3
Sept.	1/4		14.8	9.1	10.0	21.1	10.3	20.6	14.7	25.3	36.9	14.9	25.2
	1/8		4.7	0.0	0.0	8.7	7.4	9.6	5.6	10.9	12.3	8.0	9.2
	1/2			46.0	38.5	34.6	26.9	29.8	52.7	57.5	39.2	31.7	16.4
Oct.	1/4			28.1	17.0	11.7	19.6	13.2	46.9	28.3	10.8	28.5	11.9
	1/8			15.4	0.0	0.0	7.2	7.2	23.9	29.2	0.0	15.6	3.2
	1/2				26.5	19.3	35.0	36.1	39.1	49.6	27.4	27.1	10.8
Nov.	1/4				15.5	10.9	18.9	15.1	24.2	21.1	27.6	7.3	7.3
	1/8				16.7	0.0	7.8	9.6	9.1	19.0	21.2	10.0	2.9
	1/2					17.1	21.5	35.6	48.6	22.4	32.0	23.8	13.8
Dec.	1/4					11.2	13.6	17.4	24.3	12.4	18.4	12.1	11.8
	1/8					8.8	6.7	3.1	25.0	6.3	6.0	5.7	7.3
	1/2						24.1	17.6	31.9	40.8	17.2	19.5	17.4
Jan.	1/4						15.3	12.5	23.5	15.8	5.2	4.5	12.1
	1/8						0.0	8.3	12.8	9.5	4.7	3.3	4.2

TABLE 23 - Continued

Monthly Frozen Pit. Source	Pituitary Dilutions in 10 ml. Fluid	Percentage Ovulation for Various Pituitary Dilutions											
		Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July
	1/2							65.6	25.4	10.1	19.0	19.6	25.0
Feb.	1/4							22.1	15.8	3.9	4.9	15.9	16.4
	1/8							8.4	3.6	0.0	2.4	8.0	9.0
	1/2								42.7	39.2	46.5	23.9	22.4
Mar.	1/4								17.9	27.8	29.5	13.9	19.1
	1/8								4.4	5.5	11.5	0.0	11.3
	1/2									43.1	18.5	23.1	36.3
Apr.	1/4									45.1	8.3	8.9	17.9
	1/8									2.1	11.8	2.4	8.9
	1/2										26.6	29.0	42.3
May	1/4										15.0	17.6	21.0
	1/8										10.3	4.3	9.2
	1/2											22.7	53.5
June	1/4											9.9	25.6
	1/8											4.5	9.2
	1/2												38.2
July	1/4												20.4
	1/8												11.8
Monthly Mean		36.1	28.3	35.8	32.3	32.0	27.0	38.3	37.7	34.9	29.1	26.3	29.2
		21.6	16.7	19.6	17.5	17.1	17.1	18.4	23.1	21.9	16.9	16.1	16.5
		8.3	6.7	10.3	7.3	8.1	7.4	8.7	11.0	9.7	8.2	7.1	8.0

If it is true that the female spadefoot is not a seasonal breeder, it seemed necessary to determine whether the male toad was likewise capable of breeding throughout the year. Therefore, in each month from July, 1954, through June, 1955, two male toads were injected with the equivalent of two male pituitaries macerated in 1 ml. of Amphibian Ringer's Solution. Sperm being found in the urine of each animal, in every month, indicated that mature, viable sperm were present in the testes and that the male toad was almost certainly capable of fertilizing ova under natural conditions.

DISCUSSION

The question may be raised as to whether the *in vitro* ovulation technique which has been employed, not only in this study, but also by other workers, is a valid method of studying ovulation and the factors involved in this process. Heilbrunn, Daugherty, and Wilbur (1939) pointed out that eggs shed from ovaries of *Rana pipiens* and suspended in pituitary solutions showed polar body formation as do eggs ovulating naturally. In 1940, Ryan and Grant further substantiated the normality of *in vitro* ovulation by reporting that eggs, ovulated *in vitro* and passed through the oviducts of an ovariectomized female, were capable of being fertilized and producing swimming tadpoles. Wright (1945) showed that the relationship between the percentage of ovulation and the concentration of pituitary materials is the same for both the *in vivo* and the *in vitro* techniques. It has further been shown, both by Wright (1945) and by the present work, that large doses of pituitary are ineffective in producing ovulation in anurans, and this is in line with the observations of Foster, Foster, and Hisaw (1937) on the rabbit. There is a striking similarity between both the upper and the lower limiting temperatures found for the *in vitro* tests in this study, and the range of temperatures at which the toad is active and at which breeding takes place in nature. Finally, it is well known that ovulation *in vivo* is stimulated by gonadotropic factors. In the studies on the spadefoot,

using the in vitro technique, ovulation occurred only where pituitary had been used, and no spontaneous ovulation occurred in any control vials containing Holtfreter's solution. Since the parallelism between in vitro ovulation and in vivo ovulation is so great, it seems safe to conclude that the in vitro technique does afford a valid method of studying ovulation, at least in anurans.

Some differences have been observed between ovulation in vitro in the spadefoot and the ovulation process in other forms as reported by various workers. Rugh (1948) reported that the pituitary of the male Rana pipiens had only about half the potency of the female gland, although he stated that the number of glands needed to induce complete ovulation varied seasonally. In the spadefoot the writer found that the male pituitary was about 78 percent as potent as the female pituitary. The discrepancy between these potency values may be due to specific differences, since the two genera are phylogenetically well separated within the Anura. Rugh's work tested intact female frogs for normal ovulation following pituitary injections, and he was more or less concerned with an all or none response. In comparison, the present in vitro study was quantitatively more accurate, since a precise ovulation percentage could be obtained.

Again, Wright (1945), using Rana pipiens, obtained maximal ovulation in vitro with one-eighth of a pituitary in 10 ml. of fluid,

whereas one entire gland in 10 ml. of fluid was required for maximal effects in Scaphiopus holbrooki. Several factors might cause this difference. In the first place, Wright used female pituitary while male pituitary was used in the present study. But the difference in required concentrations is too great to be accounted for solely by the difference in potency of the pituitary of the two sexes. The pars distalis from a mature southern leopard frog is two or three times as large as that from an adult spadefoot toad. The pieces of ovary used in the two studies were approximately the same size, since the size of the vitellus is almost identical in Rana pipiens (1.3 - 2.0 mm.) and Scaphiopus holbrooki (1.4 - 2.0mm.). The hormone titer of the pituitary, among individuals of the same sex in a single species, seems to be pretty much the same regardless of the size of the animal or of the gland itself. However, since the pituitary of the Rana pipiens is much larger than that of Scaphiopus, it seems logical to expect that its hormone titer would therefore be a good deal higher. If this is true, then a portion of the gland should yield as much hormone as an entire gland of Scaphiopus holbrooki. This may account for the apparent difference in concentration required to induce maximal ovulation in the two species. These hormonal differences indicate that, for experimental purposes, a standard method of determining the actual hormone titer is needed. Measurements in terms of numbers or fractions of pituitaries is unsatisfactory.

A considerable difference in the time required to initiate the onset of ovulation *in vitro* was found in *Scaphiopus holbrooki* as compared to *Rana pipiens*. Wright (1945) found that *in vitro* ovulation in *Rana pipiens* started about the tenth hour, whereas in the present work it was found that ovulation in *Scaphiopus holbrooki* began in three and one-half hours. The explanation for the time differences in these studies is probably to be found in the pituitary dilutions used. Although Wright (1945) stated that none of the pituitary dilutions (ranging from one thirty-second of one pituitary to two pituitaries in 10 ml. fluid) would initiate breeding before the tenth hour, it was noted, in the present study, that heavier concentrations of pituitary initiated the ovulation response in less time. Pituitary dilution having the equivalent of four glands in 10 ml. of fluid elicited *in vitro* ovulation in one and one-half hours in *Scaphiopus*. It is possible that the ovaries of the spadefoot react to gonadotropins in a shorter time than do those of most anurans, since the entire act of breeding and embryonic development is accelerated in this form. This phenomenon is undoubtedly correlated with the habit of breeding in temporary water. Trowbridge (1941) wrote, "In *Scaphiopus bombifrons*, the rate of development throughout the entire embryonic and larval period is more rapid than that which has been reported for any other amphibian. The cleavages, in particular, take place with surprising speed; they are among the most rapid cell divisions ever recorded."

For the spadefoot toad, there is a marked similarity between the limiting and optimum temperatures for the in vitro process and ovulation and activity in nature. The limiting temperatures for the in vitro ovulation were 50° F. and 86° F.; the optimal temperature, 74° F. Pearson (1954) found that the activity of the spadefoot was greatest between 50° F. and 84° F., with maximum activity at 69° F. which he considered optimal temperature for this species. Temperature records from thirty-four spadefoot breeding choruses showed that toads bred at temperatures as low as 47° F. and as high as 83° F. with a mean of 71° F. Although induced in vivo ovulation occurred in pituitary-injected females at temperatures of 78°, 61°, and 54° F., it was inhibited at temperatures of 45° and 37° F. Apparently, the effect of cold temperatures near 50° F. inhibits the action of gonadotropic hormones in the spadefoot; this is in accord with Houssay (1954) who reported that the action of gonadotropins was retarded at temperatures between 39° and 50° F. in Bufo arenarum.

The physiological process of in vitro ovulation in the spadefoot toad has its ecological zero (i.e., "the highest externally imposed temperature at which a physiological process cannot successfully be completed." - Allee et al., 1949) at 48° F. The effective temperature threshold (i.e., "the lowest temperature at which a given physiological process can be carried through to completion." - Allee et al., 1949) for in vitro ovulation in the spadefoot was 50° F. This

concept might be extended to include the upper temperature range with the effective temperature threshold at 86° F. It was noted that the *in vitro* ovulation temperature curve closely approximated the theoretical curve representing the general effect of temperature on animal activity (Allee *et al.*, 1949). Like most physiological processes, the optimal temperature (74° F.) of ovulation *in vitro* is much nearer the point of heat coma (88° F.) than the point of cold narcosis (48° F.).

One of the physical factors tested for its effect upon *in vitro* ovulation was pH. A sodium veronal acetate buffer, used to test the pH tolerance of the *in vitro* process, produced complete inhibition of ovulation *in vitro*. The explanation for the inhibitory action of this buffer system is not clear. Since veronal, or barbital, is used as a soporific, it is entirely possible that the narcotic effects of the barbiturate inhibited the ovulation process. Houssay (1947) showed that the expulsion of the ovum from the ovarian membranes was due to compression by smooth muscles in the follicular wall. It is possible that these muscle cells were narcotized to such an extent that the eggs could not be released. Sollman (1944) stated that an adequate amount of barbiturate always depresses smooth muscles, but in varying degrees. The exact action of this narcotic is unknown. However, when a sodium phosphate buffer was used, *in vitro* ovulation progressed normally at pH values of 6.5 to 8.5, and was optimal at 7.3.

Heteroplastic pituitary materials have been used extensively for the induction of *in vivo* ovulation (Creaser and Gorbman, 1939), but the present study is the first to make a wide utilization of interspecific pituitaries for *in vitro* ovulation. The only previous *in vitro* study employing heteroplastic pituitary preparations (sheep pituitary extract) was conducted by Wright and Hisaw (1946) in *Rana pipiens*. In the present study, heteroplastic materials, from representatives of the majority of the vertebrate classes, were used, but very little could be determined quantitatively concerning the comparative amounts of pituitary required to produce equal amounts of ovulation in the various classes.

The complete negative results obtained with piscine pituitary are in accord with the majority of studies which have employed fish pituitary for the induction of *in vivo* ovulation in amphibians (Creaser and Gorbman, 1935, 1936, and Rostand, 1934). The fact, that the pituitaries from the true frogs (*Rana*) and the toads (*Bufo*), gave about the same ovulation percentages as did the controls (*Scaphiopus*), while those from the tree frogs (*Hyla*) and the narrow-mouthed toad (*Microhyla*) gave lower results, is probably due to the size of the pituitary glands from these anurans. The low percentage of ovulation using pituitary from the salamander *Desmognathus fuscus auriculatus* is in agreement with the work of Adams and Granger (1938) who found that *in vivo* ovulation in *Rana pipiens* could only be induced with

large numbers of Triturus pituitaries. Pituitary from both snake (Natrix) and lizard (Sceloporus) induced low percentages of ovulation when compared with pituitary from the control Scaphiopus, but even so, this is the first successful attempt at stimulating gonad activity in Amphibia with the reptilian pituitary. The only other study which used reptilian glands (snake - Xenodon) to induce ovulation gave negative results in Bufo arenarum (Houssay and Giusti, 1929). Bird pituitary has proved variable in inducing ovulation in Amphibia. Houssay and Giusti (1929) and Creaser and Gorbman (1939) obtained negative results when they used chicken pituitary on the toad and frog, while Stein (1934) and Witschi et al. (1937) found that avian pituitary would induce ovulation in the newt Triturus. The present study is the first to successfully utilize avian pituitary to induce ovulation in the Amura.

Creaser and Gorbman (1939) stated that those Amphibia which are responsive to mammalian pituitary preparations, require large doses to induce ovulation due to the phylogenetic, limiting factor. In the light of this statement, the sensitivity of in vitro ovulation to the majority of mammalian hypophyseal preparations is remarkable. In the present work, only two of the mammalian pituitary materials used gave negative results and there is a possible explanation for one of these. The failure of the swine gonadotropic fraction to induce in vitro ovulation might be due to the method of

preparation of the desiccated powder; possibly the protein complex in the hormone was denatured or altered in some way. Actually, this gonadotropic fraction should be more effective in eliciting ovulation in vitro than the whole pituitary or the anterior lobe preparations.

The failure of the chorionic gonadotropin, from human pregnancy urine (extract and whole urine), to initiate in vitro ovulation in Scaphiopus was not surprising, since thirteen of fifteen tests, using human pregnancy urine, were unsuccessful in inducing ovulation in eleven different species of Anura. Both Hansen (1951) and Knepton (1951) found that it was impossible to induce spermiation in the male spadefoot by the injection of human pregnancy urine or commercial human chorionic gonadotropin. The insensitiveness of both the male and female Scaphiopus to human chorionic gonadotropin may well illustrate phylogenetic specificity.

Although most naturalists recognize that the eastern spadefoot toad breeds over a broad range of months during the spring and summer, the majority imply that the breeding of this form follows a cyclic, seasonal pattern. According to the available breeding record, Scaphiopus breeds more often in the spring (57 choruses) and summer (45 choruses) than in the fall (12 choruses) and winter (7 choruses), because suitable environmental conditions (heavy rainfall and optimal temperatures) appear with greater frequency in the spring and summer.

Nevertheless, this does not negate the fact that breeding may occur at any time of the year when the temperature and rainfall permit. If extensive records had been kept, it is almost certain that spadefoot breeding would have been reported for every month in the year, but no records are available for November and December.

The range of months in which spadefoot toads have been reported to breed varies from five months (April to August) in the northern states, to six months (March to August) in the middle states and ten months (January to October) in the southern states. The differences in the range of breeding months for these three geographic areas are almost certainly due to temperature and not rainfall. Undoubtedly, the low temperatures from September to the following spring prevent fall and winter breeding in the northern, as well as, in the middle states. In the southern states, the milder temperatures seldom act as a limiting factor, and breeding occurs as early as February and as late as October.

In some portions of the spadefoot's range, there is an intensity of breeding during certain months. In the northern states, it is probable that the cyclic weather conditions are repeated with enough regularity each year to produce the preponderance of breeding which occurs in April. The spadefoots of the middle eastern states show very little fluctuation in breeding intensity for any month.

In the southern part of the range, there seems to be an increase in breeding in February and March and again in August, September, and October. The number of records for June is also high. It is possible that breeding takes place at about the same rate in each month with a decline between October and February, due to colder temperatures. The paucity of July breeding records for Florida, as well as for the other southern states, presents a problem, especially when this month has one of the highest rates of precipitation. A review of the Florida precipitation records from 1893 to 1927 showed that July had more days with rainfall than any other month of the year (Mitchell and Ensign, 1928). This would indicate that July rainfall is not as intense as the sporadic rains of other months that have equal or more total precipitation. It is during heavy sporadic rainfall that spadefoots generally breed.

It is now clear that the eastern spadefoot does not follow a seasonal breeding pattern, but breeds at any time when the proper environmental conditions prevail. Since most Amphibia resorb their eggs when unfavorable conditions exist during their usual breeding season, the retention of mature, viable eggs by the spadefoot would seem to present a physiological problem. If Scaphiopus follows the general pattern of most anurans, it is possible that there is a constant resorption of post-mature ova accompanied by a production of newly maturing eggs. It seems likely that this is the case,

since even the most mature ovaries possess some immature eggs in various stages of development. On a few occasions, it was found that ovaries from some females contained practically all immature, yolk-deficient eggs, even though breeding had not occurred for an extended period. There is the possibility that individuals resorb their eggs, according to their particular cycle, at various times of the year. There is a remote possibility that spadefoots are able to retain mature eggs until they breed, regardless of the time lapse since the last breeding, which in some cases may be several years or more. A thorough study of this problem is definitely needed.

One of the problems inherent in working with seasonal breeding in Amphibia is whether it is better to dispense with experimental work until the species may be used for breeding again, or to use two or more species which have different breeding seasons. This study has shown that the spadefoot is not a seasonal breeder, but produces mature eggs and sperm at all seasons of the year. Therefore, for embryologists, endocrinologists, and others, Scaphiopus holbrooki affords a year round supply of ovarian or embryonic material from a single species.

It appears fairly certain that breeding in the spadefoot toad is initiated by the stimulating effects of heavy rainfall and optimal temperatures. Exactly how these stimuli affect the toad is unknown. Since it is known that the ovaries are extremely sensitive

to gonadotropic secretions, it was hoped that the in vitro technique might be employed to test whether stimuli stimulating environmental conditions would cause the hypophysis to release hypersecretions of gonadotropin which sensitize the ovaries. In vitro ovulation results obtained from experimental females exposed to light, abrupt changes of temperature, and lowered atmospheric pressure, for a period of forty-eight hours, did not differ significantly from results obtained from control animals. However, experimental females exposed to water gave significantly higher ovulation percentages than controls kept in a dry state. Using a gradient of soil moistures, it was found that ovulation in vitro was correlated with water-uptake which is dependent upon the amount of soil moisture. Females subjected to a gradient of humidity chambers (98% to 0%) also showed that there was a direct correlation between ovulation results and percent humidity. The reason that ovulation was generally lower in toads exposed to a humidity gradient was that they were not in direct contact with water. It has long been known that Amphibia cannot absorb water from the air even though it may be fully saturated (Adolph, 1932).

It is well established that the antidiuretic hormone secreted by the neurohypophysis (posterior lobe) controls water balance in anurans. An increase in the osmotic pressure of the blood and body fluids stimulated the secretion of this hormone, which causes

increased water-uptake through the skin and retards the rate of urine formation by means of glomerular constriction. It is quite possible that the water regulatory function is in some way tied up with ovulation regulation. This might be the case since normal ovulation only occurs in spadefoots in which water-uptake has taken place. This may be indirect evidence that hypotonic blood and body fluids act upon the pituitary, causing it to release gonadotropins. Houssay (1949) reports that gonadotropins can be isolated from the blood at the time of breeding. In the present study it was possible to isolate only one specific environmental stimulus affecting the gonads; that is, water-uptake.

From this point, the writer would like to postulate, in accordance with experimental and field observations, on what are believed to be the steps involved in spadefoot breeding. In their burrows, spadefoots are subject to desiccation or hydration, depending upon the moisture content of the soil. Bragg (1944) suggested that the depth of the burrow is correlated with the amount of soil moisture. When rainfall is sufficient to produce a soil moisture great enough to be absorbed by the toads, water-uptake occurs and each toad's body fluids are replenished, with excess fluids being stored in the lymph sacs and bladder. These facts are in accord with recent works dealing with the relationship of anuran water economy to terrestrialism (Thorson, 1955, Ewer, 1950, and Thorson and Svihla, 1943).

Females are physiologically conditioned for breeding once the ovaries have been sensitized by water-uptake. It has also been shown that the pituitary-injected male anurans will not release sperm, even after "histological spermiation" has occurred, if insufficient body fluids prevent urine formation (Valle, Penhos, and Houssay, 1952). It seems likely, therefore, that excessive rains are needed for water-uptake by both male and female spadefoots before breeding can occur.

The actual mechanism stimulating the males to call and pairs to enter amplexus is not known. The following explanation seems plausible in the light of field observations. Both males and females have been collected from low basins and fields. At times of heavy rainfall, water collects in these areas and forms temporary ponds in which breeding choruses have been found. Males and females, having burrows in these low areas, where the ground is first saturated, are the first to be conditioned by water-uptake. Heavy rain eventually forms a temporary pond which covers the area where these burrows were located. Toads which are flooded from their burrows are probably the initiators of the breeding chorus. Since the writer has repeatedly observed that males will immediately clasp females when the two sexes are placed together in water, it seems probable that the first amplexing pairs would be those individuals which come into contact while floating at the surface of the accumulating water.

In a sense, the rapid formation of such temporary ponds is comparable to placing the spadefoot in water where amplexus is known to occur. This would seem to explain the reason why spadefoots do not migrate to permanent ponds for breeding. The breeding ponds must, figuratively speaking, be brought to them, and this is essentially what takes place when temporary ponds are formed over their burrows. Once toads are flooded from their burrows, free males utter loud, harsh, breeding calls which, according to most naturalists, stimulate both males and females to migrate to the breeding chorus.

On one occasion (February 7, 1955, Gainesville, Florida), the writer observed a small chorus (15 to 30 spadefoots) breeding in a temporary pond. Hundreds of spadefoots remained in and near their burrows on the surrounding slopes, apparently unstimulated by the call from these males. This fact probably explains how breeding can occur in a large population many times during a given year.

There is the possibility that the soil on the higher ground did not contain sufficient moisture to allow optimal water-uptake by the toads occupying these areas. Possibly spadefoots only breed when flooded from their burrows, and those living in higher areas breed only when torrential downpours occur. The fact that spadefoots do not frequent permanent ponds, even in heavy rainfall, supports the idea that they normally breed only when flooded from their burrows.

During amplexus, large secretions of gonadotropins cause individuals of each sex to liberate and expel their respective gametes: sperm in the male (spermiation) and ova in the female (ovulation).

The present study on *in vitro* ovulation in *Scaphiopus holbrooki* has shown that the natural breeding process of this form merits further investigation.

SUMMARY

1. The physiological and ecological aspects of *in vitro* ovulation of the eastern spadefoot toad, *Scaphiopus holbrooki*, were studied from May, 1954, through July, 1955.

2. A total of 1007 male and female spadefoot toads, collected near Gainesville, Florida, were used as pituitary and ovarian donors.

3. Pieces of spadefoot ovaries were suspended, on cotton threads, in stoppered vials containing pituitary materials macerated in Holtfreter's solution. Percentages of ovulation were determined by dividing the number of eggs ovulated by the total number of eggs present in the ovarian fragment and multiplying by 100.

4. Male spadefoot pituitary was found to be approximately 78 percent as potent as female pituitary in evoking ovulation *in vitro*.

5. Although individual variation in ovulation percentages was relatively high, no significant differences were obtained among individuals when a number of tests were made.

6. In experiments involving 371 separate *in vitro* tests, little or no correlation was found between the size of the male pituitary donor (in effect, the size of the pituitary) and the degree of ovulation induced.

7. No relationship was found to exist between the size of female ovarian donors and the percentage of ovulation.

8. A dilution of one pituitary suspended in 10 ml. of Holtfreter's solution evoked maximal *in vitro* ovulation, while concentrations above and below this dilution tended to produce smaller percentages of ovulation.

9. When a dilution of one pituitary in 10 ml. of solution was used, ovulation *in vitro* began in three and one-half hours and was completed after eleven and one-half hours. Ovulation was most rapid in the three hours immediately following its initiation, with a maximal peak during the third hour.

10. Light and darkness had no apparent effect on *in vitro* ovulation.

11. *In vitro* ovulation occurred between 10° and 30° C., with an optimum temperature at about 24° C. Complete inhibition occurred at 9° and 31° C.

12. Ovulation *in vitro* occurred between pH values of 6.5 and 8.5 in a 0.07M sodium phosphate buffering system. Maximal activity took place near the point of neutrality, at a pH of 7.3. The complete inhibition of ovulation resulting from the use of a veronal acetate buffer was probably due to the narcotic effects of the veronal.

13. Heteroplastic pituitary materials from representatives of most of the vertebrate classes were used in the induction of ovulation *in vitro*. Amphibian, reptilian, avian, and the majority of mammalian pituitary preparations evoked ovulation, while piscine pituitary, swine gonadotropin fractions, and human chorionic gonadotropins

reacted negatively. The present study was the first to successfully utilize reptilian and avian pituitaries for the induction of ovulation in the Anura.

14. A study of available records indicated that breeding in the spadefoot does not follow a cyclic, seasonal pattern, but may occur in almost any month of the year. Breeding appears to be initiated by a combination of heavy rainfall and warm temperature.

15. Using the in vitro ovulation technique it was possible to test for ovarian sensitization. Exposure of females to light and darkness, abrupt changes of temperature, and lowered atmospheric pressure produced no apparent effect upon in vitro ovulation. However, when females were placed in contact with water or exposed to high humidities, significantly higher percentages of in vitro ovulation were obtained. It has been suggested that water-uptake may stimulate the hypophysis to release gonadotropins which subsequently sensitize the ovaries.

16. Hypophysectomy of female toads caused a sensitization of the ovaries which resulted in higher in vitro ovulation percentages.

17. A monthly study of spadefoot gonads revealed no seasonal variation in the condition of either the ovary or the testis.

18. Because of the marked parallelism between ovulation in vitro and ovulation in vivo, it was concluded that the in vitro technique afforded a valid method of studying amuran ovulation.

19. It has been observed that spadefoots are active in nature between 50° and 84° F. with an optimum of 69° F., while breeding occurs between 47° and 83° F. with an optimum of 71° F. The marked similarity between these temperatures and the limiting (50° - 86° F.) and optimum (74° F.) temperatures for in vitro ovulation lends further support to the validity of the in vitro technique.

20. Since the majority of spadefoot toads retain mature ova and sperm throughout all seasons, they afford an excellent source of ovarian and embryonic material for the embryologist, endocrinologist, and others.

21. Under natural conditions, water-uptake is necessary to the female for ovarian sensitization and for urine formation which provides a medium for the transportation of sperm in the male. It is therefore suggested that toads which are flooded from their burrows in the low areas probably are the first to initiate the breeding chorus.

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PLATE I

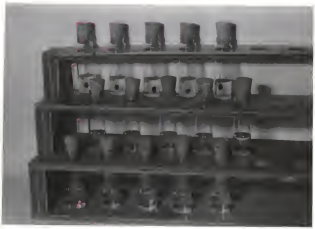
Fig. 1. Single excised spadefoot ovary containing nine egg-filled lobes.

Fig. 2. Vial-rack holding vials which contain ovarian fragments suspended on cotton threads in pituitary extract.

Fig. 3. Control vial (left) containing only Holtfreter's solution and experimental vial (right) containing Holtfreter's solution and pituitary homogenate. Eggs at the bottom of the experimental vial have been ovulated from the hanging ovarian fragment.

PLATE I

1.



2.

3.

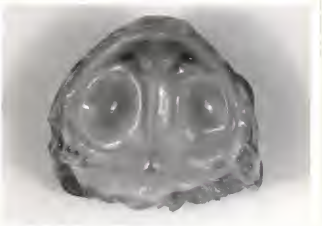
PLATE II

Fig. 1. Ventral view of excised spadefoot cranium severed through the cervical region and angle of the jaw.

Fig. 2. Cranium showing exposed parasphenoid bone after the removal of the oral mucosa from the roof of the mouth.

Fig. 3. Cranium with brain case opened ventrally, showing the exposed pituitary gland (small white lobe) just posterior to the optic chiasma.

PLATE II



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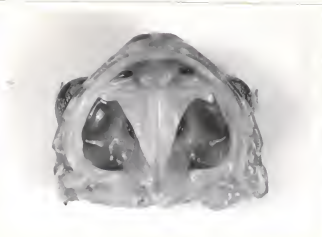

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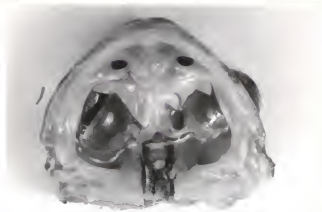

3.

BIOGRAPHICAL SKETCH

Keith Leyton Hansen, born November 14, 1925, in Gainesville, Florida, attended schools in Jacksonville, Florida. After graduation from Andrew Jackson Senior High School in 1943, he attended the University of Florida for one semester. From March, 1944, to July, 1946, he served in the United States Army. He entered John B. Stetson University in 1946. He was married to the former Mary Juanita Turner of Jacksonville, Florida, in August, 1947. Having graduated from Stetson University with a Bachelor of Science degree Cum Laude in August, 1949, he began graduate studies in September, 1949, and received a Master of Science degree in August, 1950. While at Stetson University, he served as a graduate assistant. He was employed as an Instructor in biology from September, 1950, to June, 1951. Then he began graduate studies at the University of Florida. While attending the University of Florida, he was employed as a graduate assistant until August, 1954. Then he was awarded a graduate fellowship until August, 1955.

He holds membership in Florida Academy of Sciences, Herpetologists League, American Society of Ichthyologists and Herpetologists, American Association for the Advancement of Science, Beta Beta Beta, Kappa Delta Pi, Phi Sigma, and Sigma Xi.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of the committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 13, 1955

C. I. Byers
Dean, College of Arts and Sciences

Dean, Graduate School

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